

**TABLE 4**  
**Biophysical properties of  $\alpha_{1H}$ -containing  $Ca^{2+}$  channels**

Properties:	$\alpha_{1H}$ HEK293	$\alpha_{1H}$ <i>Xenopus</i> Oocytes	Native T-type <sup>b</sup>
5 Relative conductance conductance [pS]	$Ba^{2+} \cong Ca^{2+}$ $9.06 \pm 0.22$	$Ba^{2+} \cong Ca^{2+}$ n.d.	$Ba^{2+} \cong Ca^{2+}$ 5-9
Activation			
kinetics, $\tau$ [ms]	$2.8 \pm 0.5^c$	$3.3 \pm 0.5^c$	2 to 8
10 $V_{1/2}$ [mV]	$-25.1 \pm 3.9$ $25.5 \pm 9.9$	n.d.	-60 to -45
Inactivation			
kinetics, $\tau$ [ms]	$16.9 \pm 5.3^c$ $-63.2 \pm 2.0$	$23.3 \pm 1.5^c$ $-64.5 \pm 1.0$	10 to 30 -100 to -50
15 $V_{1/2}$ [mV]	$0.64 \pm 0.21$	n.d.	2 to 12
Tail deactivation $\tau$ [ms]	$2.1 \pm 1.06$		

<sup>b</sup> Huguenard (1996) Annual Rev. Physiol. 58:329-348; <sup>c</sup> determined at -20 mV test potential; n.d. not determined

## 20 E. Properties of calcium channels containing $\alpha_{1H-2}$ subunits

### Summary Discussion

The biophysical properties of  $\alpha_{1H-2}$ , revealed a shift in the  $V_{1/2}$  of isochronic inactivation (20 seconds) to -73 mV compared to a  $V_{1/2}$  of -62.5 mV for  $\alpha_{1H-1}$ . The  $V_{1/2}$  of  $\alpha_{1H-2}$ , thus exhibits a range closer to  $V_{1/2}$  values reported for certain native T-type calcium channels (Huguenard (1996) Annual Rev. Physiol. 58:329-348). For example, under similar recording conditions the  $V_{1/2}$  of isochronic inactivation for T-channels in rate dorsal horn neurons (DHN) is reported to be -82 mV, while the  $V_{1/2}$  recorded in rate dorsal lateral geniculate neurons (LGN) is -64 mV. In addition, the  $V_{1/2}$  of  $\alpha_{1H-2}$  more closely approximates the  $V_{1/2}$  in native rat DHN compared to the value for  $\alpha_{1H-1}$ , which, instead, comes closer to the value recorded for T-type calcium channels in LGN. Thus, the observed differences the amino acid sequence of the  $\alpha_{1H-1}$  and  $\alpha_{1H-2}$  subunits appears linked to differences in tissue distribution of these two different forms of the  $\alpha_{1H}$  channel. These results also provide basis for

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In addition, each of Amiloride and Mibefradil blocked low-threshold  $\text{Ba}^{2+}$  currents in oocytes in a concentration-dependent manner giving a calculated  $\text{IC}_{50}$  of  $161\mu\text{M}$  for Amiloride; mean of 7 experiments,  $n_H=0.62$ ) and mean of  $2.1\mu\text{M}$  for Mibefradil; mean of 4 experiments,  $n_H=0.71$ ).

- 5        These results demonstrate that incorporation of an  $\alpha_{1H}$  subunit into functional calcium channels in the membranes of cells, conveys the electrophysiologic and biophysical properties of low-voltage activated, particularly T-type, calcium channels upon those channels. The  $\alpha_{1H}$ -containing channels were activated rapidly at relatively negative
- 10       membrane potentials (i.e.,  $V_{1/2}=64.5\text{ mV}$ ), and were also inactivated rapidly (i.e.,  $\tau=12.2\text{ ms}$  at  $-20\text{mV}$ ). Peak channel open activity was observed at a membrane potential of  $-30\text{mV}$ . These channels also exhibited approximately equal permeability for  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$ .

- Pharmacologic properties of  $\alpha_{1H}$  containing channels were also
- 15       consistent with those of other low-threshold calcium channels. They are blocked by  $\text{Ni}^{2+}$  ( $\text{IC}_{50}=6.3\mu\text{M}$ ),  $\text{Cd}^{2+}$  ( $\text{IC}_{50}=10.3\mu\text{M}$ ), Amiloride ( $\text{IC}_{50}=16.1\mu\text{M}$ ) and Mibefradil ( $\text{IC}_{50}=2.1\mu\text{M}$ ).

- D.       Comparison of calcium channels containing human  $\alpha_{1H}$  subunits expressed in HEK293 Cells with those expressed in *Xenopus*
- 20       oocytes

TABLE 4 summarizes the biophysical properties of: (i) human  $\alpha_{1H-1}$ -containing calcium channels expressed in HEK293 cells, (ii) human  $\alpha_{1H-1}$ -containing channels expressed in *Xenopus* oocytes, and (iv) native T-type calcium channels expressed in various tissues.

relatively electro-negative activation range of  $\alpha_{1H}$  channels in comparison with the high-voltage activated  $\alpha 1A-2\alpha 2b\delta\beta 4a$ ,  $\alpha 1B-1\alpha 2b\delta\beta 3a$  and,  $\alpha 1E-3\alpha 2b\delta\beta 1b$  calcium channels.

5           **3. Permeability, Inactivation and Biophysical Properties of Human  $\alpha_{1H}$  Expressed in *Xenopus* oocytes**

Permeability and inactivation properties of human  $\alpha_{1H}$  channels were investigated in oocytes by studying  $Ba^{2+}$  and  $Ca^{2+}$  currents. The results show that  $Ba^{2+}$  currents were not significantly larger than  $Ca^{2+}$  currents in oocytes expressing the  $\alpha_{1H}$  subunit. Results presented in 10 show normalized steady-state inactivation curves for  $\alpha_{1H}$ -mediated  $Ba^{2+}$  currents, where  $V_{1/2}$  was calculated to be equal to a value of  $-64.5 \pm 1.0$  mV. A double pulse protocol, i.e., with increasing time intervals between pulses, was used to examine the recovery of  $\alpha_{1H}$  channels from inactivation. The results of relative recovery of channels plotted against 15 the interpulse interval (ms) and demonstrated that  $\alpha_{1H}$  channel currents recovered quickly from inactivation, with an average time constant of 330 ms ( $n=5$ ).

**4. Cadmium, Nickel, Amiloride and Mibefradil Antagonize human  $\alpha_{1H}$  Channel  $Ba^{2+}$  Currents**

20            $Cd^{2+}$  was found to antagonize low-threshold human  $\alpha_{1H}$  currents in oocytes in a concentration dependent manner. By plotting the inhibition of  $Cd^{2+}$  as the percentage of the control  $Ba^{2+}$  current achieved at different concentration of  $Cd^{2+}$ , an  $IC_{50}$  of  $10.3\mu M$  as calculated.  $Ni^{2+}$  was also found to antagonize low-threshold human  $\alpha_{1H}$  channels in 25 oocyte, and also in a concentration dependent manner. The inhibition of  $Ba^{2+}$  currents produced by different concentrations of  $Ni^{2+}$  ( $n=4$  experiments;  $n_H=0.84$ ) was tested. The calculated  $IC_{50}$  for  $Ni^{2+}$  was  $6.3\mu M$ . Antagonism by  $Ni^{2+}$  and  $Ba^{2+}$  were largely reversible.

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approximately -50 mV with peak responses between -30 mV and -40 mV, which is consistent with low voltage activated channels. Permeability of the  $\alpha_{1H}$  channels to  $\text{Ca}^{2+}$  was slightly greater than to  $\text{Ba}^{2+}$ . In contrast with high voltage channel, the  $\alpha_{1H}$  channels activated slowly ( $\tau = 5.7 \pm$   
5 1.0 ms at the peak of the I-V curve,  $3.3 \pm 0.5$  ms at -20mV) and inactivated rapidly ( $\tau = 13.4 \pm 1.9$  ms at the peak of I-V curve,  $12.2 \pm 1.5$  ms at -20 mV). The  $\alpha_{1H}$  channels expressed in oocytes were sensitive to steady-state inactivation at relatively negative membrane potentials ( $V_{1/2} = -64.5 \pm 1.0$  mV) and recovered quickly from inactivation ( $\tau$  of  
10 recovery  $\approx 330$  ms). These values are very similar to those obtained from  $\alpha_{1H}$  channels expressed in HEK293 cells. The  $\text{Ba}^{2+}$  currents through  $\alpha_{1H}$  channels in oocytes were sensitive to blocking by  $\text{Ni}^{2+}$  and  $\text{Cd}^{2+}$  with IC50 values of  $6.3\mu\text{M}$  and  $8.3\mu\text{M}$ , respectively. Of the antagonists tested, only amiloride ( $\text{IC}_{50} \approx 16\mu\text{M}$ ) and mibefradil ( $\text{IC}_{50} \approx 2\mu\text{M}$ ) markedly  
15 inhibited  $\alpha_{1H}$ -mediated  $\text{Ba}^{2+}$  currents through  $\alpha_{1H}$  channels expressed in oocytes. Taken together the results indicate that  $\alpha_{1H}$  represents a low-voltage activated calcium channel subunit.

## 2. Activation and Inactivation Properties of $\alpha_{1H}$ Channel $\text{Ba}^{2+}$ Currents

20 Current-voltage relationships for  $\text{Ba}^{2+}$  (15 mM) currents were recorded from single oocytes injected with mRNA encoding the human  $\alpha_{1H}$  subunit.  $\text{Ba}^{2+}$  currents were activated at a membrane potential of about -50 mV and peaked at -30 mV. The relative inactivation rates of human  $\alpha_{1H}$  channels were investigated in different oocyte preparations and  
25 compared with inactivation rates of  $\alpha 1A-2\alpha 2b\delta\beta 4a$  channels;  $\alpha 1B-1\alpha 2b\delta\beta 3a$  channels; and,  $\alpha 1E-3\alpha 2b\delta\beta 1b$  channels.  $\text{Ba}^{2+}$  currents were elicited using a voltage command in the range of -120 mV to -30 mV for  $\alpha_{1H}$  channels, or -90 mV to 0 mV or +10 mV for the other respective  $\alpha_{1A}$ ,  $\alpha_{1B}$  and  $\alpha_{1E}$  containing channels. The results presented show the



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## 5. Recovery from Inactivation

Recovery of  $\alpha_{1H}$  channels expressed transiently in HEK293 cells from inactivation induced by using a double pulse protocol using depolarizing pulses to -20mV was evaluated. The fraction of recovered channels was plotted vs. interpulse interval and the data point were fitted by a bi-exponential function in the form  $I = A_0 + A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$ .  $\tau_1$ :35 ms,  $A_1$ :0.165,  $\tau_2$ :337 ms,  $A_2$ :0.788.

## 6. Single-Channel Recording from Human $\alpha_{1H}$ calcium channels

Single-channel properties of  $\alpha_{1H}Ca^{2+}$  channels in HEK293 cells were determined in cell-attached recordings with 110 mM  $Ba^{2+}$  as the charge carrier. Single-channel recordings at a test potential of -30 mV from a patch that contains at least three  $\alpha_{1H}$  showed that channel openings occurred in bursts and were clustered mainly in the first third of the 100-ms depolarizing pulse, especially with stronger depolarizations. Occasionally, channel activity was spread throughout the entire sweep. The time course of the ensemble-averaged current recorded at -30mV in 110 mM  $Ba^{2+}$  was similar to the  $\alpha_{1H}$  whole-cell  $Ba^{2+}$  current recorded at -40 mV in 15 mM  $Ba^{2+}$ . The currents were compared at different potentials to compensate for the shift in the activation curve to more positive potentials due to the increase in divalent concentration. The unitary current-voltage relationship yielded a unitary slope conductance of  $9.06 \pm 0.22$  pS ( $n=4$ ).

## C. Biophysical Characterization of Human $\alpha_{1H}$ calcium channels in *Xenopus* Oocytes

### 1. Overview

Cloned human  $\alpha_{1H}$  calcium channels were characterized further by transient expression of  $\alpha_{1H.1}$  mRNA in *Xenopus* oocytes. Injection of  $\alpha_{1H.1}$  mRNA alone resulted in expression of large currents, i.e., typically  $> 1\mu A$  when recording in 15 mM  $Ba^{2+}$ . The  $\alpha_{1H}$  channels were activated at

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the decay rate of the slow component, comprising  $88.1 \pm 33.8\%$  of the total current, was  $2.1 \pm 1.06$  ms ( $n=6$ ), which is similar to those observed in native T-type  $\text{Ca}^{2+}$  channels. The decay rate of the faster component was  $0.64 \pm 0.21$  ms ( $n = 6$ ). Slow decay of  $\alpha_{1H-1}$ -mediated tail currents were observed over a period of 15 ms.

The voltage dependence of activation of  $\alpha_{1H-1}$  containing  $\text{Ca}^{2+}$  channels was determined from tail-current analysis. Normalized tail-current amplitudes were plotted as a function of test potential and revealed a biphasic activation curve that was well fitted by the sum of two Boltzmann functions (Figure 1). The potentials for half-maximal activation of the individual Boltzmann terms were as follows:  $V_{\frac{1}{2},A}$ :  $-25.1 \pm 3.3$  mV; and  $V_{\frac{1}{2},B}$ :  $+25.5 \pm 3.9$  mV ( $n=11$ ). A value similar to  $V_{\frac{1}{2},A}$  has been reported previously for voltage dependence of activation of T-type  $\text{Ca}^{2+}$  channels in the human TT cell line ( $-27$  mV). The value of the second Boltzmann term  $V_{\frac{1}{2},B}$  is somewhat similar to that reported for HVA  $\text{Ca}^{2+}$  channels. Using a similar protocol, tail currents of HVA  $\text{Ca}^{2+}$  channels decay with time constants of  $<300$   $\mu\text{s}$ , whereas with  $\alpha_{1H}$  the most prominent at test potentials close to  $V_{\frac{1}{2},B}$ . The availability of  $\alpha_{1H}$  containing  $\text{Ca}^{2+}$  channels for opening was dependent on the membrane potential as shown in FIGURE 1. The potential for half-maximal steady-state inactivation ( $V_{\frac{1}{2}}$ ) was  $-63.2 \pm 2.0$  mV ( $n = 9$ ).

#### 4. Kinetics of Activation and Inactivation of $\alpha_{1H}$ Channels

FIGURE 2 shows the kinetics of activation (FIGURE 2A) and inactivation (FIGURE 2B) of human  $\alpha_{1H}$  calcium channels. Kinetics of activation and inactivation were determined from current traces by fitting an exponential function to rising (FIGURE 2A) or declining (FIGURE 2B) phase of the current. The voltage-dependence for activation and inactivation follows approximately an exponential function.

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## 2. Voltage-Dependence of Activation and Inactivation

FIGURE 1 shows the voltage-dependence of activation ( $m_{\infty}$ ) and steady-state inactivation ( $h$ ) of human  $\alpha_{1H}$  calcium channels expressed transiently in HEK cells. Voltage-dependence of activation ( $m_{\infty}$ ) was determined from tail current analysis. Tail currents were normalized with respect to the maximum peak tail current obtained at +60 mV and were plotted (open symbols, mean  $\pm$  SEM;  $n = 11$ ) vs. test potential. Data were fitted by the sum of two Boltzman function  $m_{\infty} = F_A * [1 + \exp(-(V_{\text{test}} - V_{1/2,A})/k_A)]^{-1} + F_B * [1 + \exp(-(V_{\text{test}} - V_{1/2,B})/k_B)]^{-1}$ ,  $F_A = 0.67$ ,  $V_{1/2,A} = -21.5$  mV,  $k_A = 7.5$ ,  $F_B = 0.33$ ,  $V_{1/2,B} = 25.5$  mV,  $k_B = 14.7$ . Steady-state inactivation ( $h_{\infty}$ ) was determined from a holding potential of -100 mV by a test pulse to -20 mV (p1), followed by a 20 second prepulse from -100 mV to -10 mV in 5 mV decrements (pHold) preceding a second test pulse to -20 mV (p2). Normalized current amplitudes were plotted (closed symbols, mean  $\pm$  SEM;  $n = 9$ ) vs. holding potential. Data were fitted by a Boltzman function  $h_{\infty} = [1 + \exp((V_{\text{hold}} - V_{1/2})/k)]^{-1}$ ,  $V_{1/2} = -63.9$  mV,  $k = 3.9$  mV.

## 3. Tail Current Deactivation

Tail current deactivation profiles for  $\alpha_{1H-1}$  calcium channels in transiently transfected HEK cells were studied. One hallmark of LVA channels is their slow rate of deactivation, which is reflected in a slow decay of tail currents. The time constant of this decay is  $\sim 10$ -fold slower for LVA channels (2-12 ms) than for HVA channels  $< 300 \mu s$ . A slow decay of  $\alpha_{1H-1}$  mediated tail currents over a period of  $\sim 15$  ms was observed. In contrast to the monoexponential decay of the tail currents reported for many native T-type  $Ca^{2+}$  channels, tail currents from  $\alpha_{1H-1}$  channels showed a biexponential decay. At a test potential of -20 mV,

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## B. Electrophysiology

### 1. Current-Voltage Properties

The rapid inactivation of  $\alpha_{1H-1}$   $\text{Ca}^{2+}$  channels was strongly voltage-dependent. The current decay was best described with an exponential function with time constants ranging from  $42.2 \pm 7.8$  to  $8.8 \pm 3.8$  ms at membrane potentials between -50 and +30 mV ( $n = 6$ ; data not show). Activation kinetics of  $\alpha_{1H-1}$   $\text{Ca}^{2+}$  channels were also voltage-dependent with time constants ranging from  $9.9 \pm 4.7$  to  $0.9 \pm 0.3$  ms for membrane potentials between -50 and +30 mV ( $n = 8$ ; data not shown).  $\alpha_{1H-1}$   $\text{Ca}^{2+}$  channels inactivated completely during the 150-ms depolarization. Recovery from inactivation occurred within a period of  $\sim 3$  s with a fast component ( $\tau = 37 \pm 9$  ms;  $16.5 \pm 4.6\%$  of all channels) and a slow component ( $\tau = 37 \pm 61$  ms;  $78 \pm 8.5\%$  of all channels;  $n = 3$ ; data not shown). To confirm the biophysical properties of recombinant  $\alpha_{1H}$  channels observed in whole-cell recordings from HEK293 cells, the functional expression of  $\alpha_{1H}$  in *Xenopus* oocytes was tested. Substantial currents ( $< 1 \mu\text{A}$ ) after injection of  $\alpha_{1H}$  transcripts alone was observed.

The current-voltage relationship for  $\text{Ba}^{2+}$  or  $\text{Ca}^{2+}$  from traces determined. Following transient transfection of HEK293 cells with a DNA encoding the  $\alpha_{1H-1}$  subunit,  $\text{Ba}^{2+}$  currents that were rapidly activating and inactivating were observed.  $\text{Ba}^{2+}$  currents (15 mM) elicited by step depolarizations to various test potentials from a holding potential of -90 mV were measured. Currents were activated at a test potential of -50 mV, peaked between -20 and -10 mV, and reversed at a membrane potential more positive than +60 mV. Similar results were obtained with  $\text{Ca}^{2+}$  (15 mM) as the charge carrier.

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***Xenopus* oocyte studies:** *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, Wisconsin). Oocytes were incubated in  $\text{Ca}^{2+}$ -free solution containing 88 mM NaCl, 1 mM KCl, 0.82 mM  $\text{MgSO}_4$ , 2.4 mM  $\text{NaHCO}_3$ , 10 mM Hepes and 1.5 mg/ml collagenase A (Worthington, Freehold NJ; Type 4, 1.5 hr and subsequently Sigma, St. Louis, MO, Type 1A, 0.5 hr.). Following collagenase treatment, oocytes were transferred to frog Ringer's solution that contained 88mM nACl, 1mM KCl, 0.91 mM  $\text{CaCl}_2$ , 0.82 mM  $\text{MgSO}_4$ , 0.33 mM  $\text{Ca}(\text{NO}_3)_2$ , 2.4 mM  $\text{NaHCO}_3$  and 10 mM Hepes. Under these conditions, manual removal of the follicle cell layer was not required. Oocytes were injected with 50 ng (1 $\mu\text{g}/\text{ml}$ ) of *in vitro* transcripts encoding the  $\alpha_{1H}$  subunit and incubated for 3-5 days at 19°C prior to recording. The incubation medium was frog Ringer's solution containing penicillin/streptomycin (Sigma; 10 ml/L), gentamicin (Sigma; 1 ml/L and 5% heat-inactivated horse serum (Gibco, Gaithersburg, MD). Microelectrodes were pulled on a horizontal puller (Model P80, Sutter Instruments, Novato, CA); filled with 3 M KCl; and selected for resistances in the range of 0.5-2.0 M $\Omega$ . Data were recorded using a GeneClamp 500; digitized at 1-5 KHz; and stored on magnetic disks for analysis offline using pClamp or Axograph software (Axon Instruments).  $\text{Ba}^{2+}$  or  $\text{Ca}^{2+}$  currents were recorded in a solution containing 36 mM TEA-OH, 2.5 mM KOH, 75 mM mannitol, 10 mM HEPES and 15 mM  $\text{Ba}(\text{OH})_2$  or  $\text{Ca}(\text{OH})_2$ , respectively at pH 7.3. Currents were leak-subtracted using the P/6 protocol. To block  $\text{Ca}^{2+}$ -activated chloride currents, niflumic acid (300 $\mu\text{M}$ ) was included in experiments where the relative permeability of  $\alpha_{1H}$  channels to  $\text{Ba}^{2+}$  or  $\text{Ca}^{2+}$  was measured. All values are reported as mean  $\pm$  S.D. unless stated otherwise. Drugs (above) were applied via a gravity-fed perfusion system. At the concentrations used herein, solvents had no effect on  $\alpha_{1H}$ -mediated currents.

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from borosilicate glass (TW150, WPI, Sarasota, FL), coated with Sylgard (Dow Corning Midland, MI), and had a resistance of 1.1-2.0 M $\Omega$  when filled with internal solution. Series resistance was 2-5 M $\Omega$  and 70-90% series resistance compensation was generally used. The pipette solution contained (in mM): 135 CsCl, 10 EGTA, 1 MgCl<sub>2</sub>, 10 HEPES (pH 7.3, adjusted with Cs-OH). The external solution contained (in mM): 15 BaCl<sub>2</sub> or CaCl<sub>2</sub>, 150 Choline Cl, 1 MgCl<sub>2</sub>, 5 TEA-OH and 10 HEPES (pH 7.3, adjusted with HCl). Single channel recordings were obtained using the cell-attached configuration of the patch-clamp technique. The pipette solution contained (in mM): 110 BaCl<sub>2</sub>, 10 HEPES (pH 7.3, adjusted with TEA-OH). The membrane potential of individual HEK293 cells was set to zero with a solution containing (in mM): 140 K-aspartate, 5 EGTA, and 10 HEPES (pH 7.3). Membrane potentials in the single channel recordings were not corrected for liquid junction potential offset (+12 mV). Linear leak and residual capacitive currents were on-line subtracted using a P/4 protocol (whole-cell recording) or scaled single-channel sweeps with no activity (single-channel recordings).

**Drugs:** Mibefradil (Ro 40-5967) was a gift from F. Hoffman-LaRoche. Nimodipine and (-)-BayK-8644 were obtained from Research Biochemicals (Natick, MA). The peptide toxins  $\omega$ -CgTx GVIA (conotoxin) and  $\omega$ -CmTx MVIIC (conotoxin) were obtained from Bachem (Torrance, A). All remaining compounds were obtained from Sigma. Stock solutions were prepared in dimethyl sulfoxide (amiloride, nimodipine), ethanol ((-)-BayK-8644) or water (verapamil, mibefradil, ethosuximide,  $\omega$ -CmTx GVIA and  $\omega$ -CmTx MVIIC) and stored at 4°C. Drugs were prepared fresh on each experimental day from stock solutions and applied via peristaltic pump at a flow rate of <0.5 ml/min. The maximal solvent concentration in the final test solution was <0.1%. At these concentrations these solvents have no effect on  $\alpha_{1H}$ -mediated currents.

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**EXAMPLE 3:        Biophysical and Pharmacological properties of  
                      channels containing  $\alpha_{1H-1}$  and  $\alpha_{1H-2}$  subunits**

**A.        Materials and Methods**

Materials and methods for biophysical and pharmacology study of  
5 calcium channel subunits are described in this EXAMPLE and EXAMPLE 4  
below with reference to previously cloned subunits. Such methods or  
other similar methods known to those of skill in the art have been used to  
study these properties of human  $\alpha_{1H-1}$  subunits as described in this  
Example.

10        **Electrophysiology:** HEK293 cells were transiently transfected with  
6  $\mu$ g pcDNA1 $\alpha_{1H}$ RBS using a standard  $Ca^{2+}$  phosphate procedure (see,  
*e.g.*, EXAMPLE below, see, also Williams *et al.* (1992) *Neuron*, 8:71-84,  
for transfection procedure). pCMVCD4, a human CD expression plasmid,  
was included in the transfections as a marker to permit the identification  
15 of transfected cells. Prior to recording, cells were washed with  
mammalian Ringer's solution, incubated for approximately 10 min in a  
solution containing a 1/1000 dilution of M-450 CD4 Dynabeads (Dyna-  
l Inc., Lake Success, NY) and rewashed with mammalian Ringer's solution  
to remove excess beads. Functional expression of  $\alpha_{1H}$  channels in  
20 transfected cells was evaluated 24-48 hours following transfection using  
the whole-cell patch clamp technique. All recordings were performed on  
single cells at room temperature (19-24°C). Whole-cell currents were  
recorded using an Axopatch-200A (Axon Instruments, Foster City, CA) or  
anEPC-9 (HEKA elektronik, Lambrecht, Germany) patch clamp amplifier,  
25 low-pass filtered at 1 kHz (-3 dB, 8-pole Bessel filter) and digitized at a  
rate of 10 kHz, unless otherwise stated. Pipettes were manufactured

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SEQ ID No. 16 shows the nucleotide sequence of the  $\alpha_{1H-2}$  splice variant. The coding sequence for  $\alpha^{1H-2}$  begins at 249 and ends at 6353.

#### B. Summary

Nucleic acid clones encoding full length  $\alpha 1H$  T-type channel subtype were isolated from TT cells. Although similar in overall nucleotide sequence topography to other previously cloned HVA  $\alpha_1$  subunits, the  $\alpha_{1H}$  subunit contained several unusual features, including a large II-III domain loop, absence of the common  $\alpha_1$  interaction domain, and altered ion selectivity properties. Two isoforms of  $\alpha_{1H}$  designated  $\alpha_{1H-1}$  and  $\alpha_{1H-2}$  were identified. The first  $\alpha_{1H-1}$  is the larger of the two, and the second  $\alpha_{1H-2}$  is the smaller of the two containing a 957 nucleotide deletion in the II-III loop relative to  $\alpha_{1H-1}$ . The nucleotide sequence of  $\alpha_{1H-1}$  is set forth in SEQ ID No. 12 and No. 15 and that of  $\alpha_{1H-2}$  is set forth in SEQ ID NO. 16.  $\alpha_{1H-2}$  contains a 957 nucleotide deletion relative to  $\alpha_{1H-1}$  which results in a loss of 319 amino acids (amino acids 470-788 of  $\alpha_{1H-1}$ ) from within the intracellular loop between domains II and III. The splice variant deletion was identified by PCR in all cells and tissues examined. These include TT-cells, amygdala, caudate nucleus, putamen, heart, kidney and liver cells. In the brain expression is primarily in the amygdala, caudate nucleus and putamen. Liver, kidney and heart have high levels. The coding sequence for  $\alpha 1H-1$  begins at nucleotide 249 and ends at nucleotide 7310 while the coding sequence for  $\alpha_{1H-2}$  begins at nucleotide 249 and ends at nucleotide 6353.

Polyclonal antiserum was raised to the putative II-III intracellular loop domain of the  $\alpha 1H$  subunit. Following transient expression in HEK293 cells a protein of the appropriate size was detected by SDS-PAGE and Western blotting. Functional characterization of human  $\alpha_{1H}$  channels is provided in EXAMPLE 3.



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degenerate amplification reaction followed by Primer-3 and Primer-2 in a nested PCR amplification. This resulted in amplification of a 340 nucleotide fragment that encodes a portion of the  $\alpha_{1H}$  subunit. This amplification product was used as a probe to screen the library to isolate  
5 nucleic acid clones encoding a full-length  $\alpha_{1H}$  subunit.

Using a primer base on the  $\alpha_{1H-1}$  sequence and RT-PCR on various tissues, transcripts with an in-frame deletion relative to  $\alpha_{1H-1}$  were identified and isolated from the TT cell library. Fragments spanning this deletion were isolated and, when lined up matched the  $\alpha_{1H-1}$  sequence  
10 except for a 957 base pair deletion. A full-length clone, designated  $\alpha_{1H-2}$  (see SEQ ID NO. 16), was constructed from among these fragments, and inserted in the pcDNA1 with the RBS as for  $\alpha_{1H-1}$ .  $\alpha_{1H-2}$  transcripts were identified in all tissues examined.

Nucleic acid encoding  $\alpha_{1H-2}$  results from an alternately spliced RNA  
15 and has a 957 nucleotide in-frame deletion relative to  $\alpha_{1H-1}$ , as detected in the PCR products from numerous tissues and cells, including TT cellular cDNA,, amygdala cDNA, caudate nucleus cDNA, putamen cDNA, heart cDNA, kidney cDNA and liver cDNA. PCR primers were: (i) 5'-primer corresponding to the sense strand of  $\alpha_{1H-1}$  at nucleotide 1373 through  
20 1393; (ii) 3'-primer corresponding to the antisense strand of  $\alpha_{1H-1}$  at nucleotide 2657 through 2680.

SEQ ID Nos. 12 and 15 show the nucleotide sequence of  $\alpha_{1H-1}$ . The coding sequence for  $\alpha_{1H-1}$  begins at nucleotide 249 and ends at 7310. (SEQ ID Nos. 12 and 15 differ in minor respects,  
25 e.g., amino acid 2230 (bases 6983-6985) is Asp (GAC) in the SEQ ID No. 15 and Glu (GAA) in SEQ ID No. 12).

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in clone 1.305 with clone 1.205 using a common EcoRI site (nt 2433 to 2438 SEQ ID No. 49); and 2) the resulting clone, which encodes the amino terminus of  $\alpha_{1H}$  was combined with the carboxyl terminal sequences of  $\alpha_{1H}$  encoded in clone 1.203 using the common EcoRV restriction endonuclease site shared between clone 1.205 and 1.203 (nt 4517-4522 of SEQ ID NO. 12). The resulting full-length human  $\alpha_{1H}$  calcium channel subunit is 2,353 amino acid residues in length (SEQ ID NO. 12). The expression construct was assembled in pCDNA1 (Invitrogen, San Diego, CA) and included a consensus ribosome binding site (RBS) followed by the full-length  $\alpha_{1H}$  coding sequence (see, for a description of pCDNA1-based vectors containing the RBS, see, *e.g.*, in International PCT application No. PCT/US94/09230, see, also allowed U.S. application Serial No. 08/149,097, U.S. Patent No. 5,851,824, and U.S. Patent No. 5,846,756). The resulting construct was designated pCDNA1 $\alpha_{1H}$ RBS.

**EXAMPLE 2: Cloning of human calcium channel  $\alpha_{1H-2}$  subunit**

T-type channel currents are heterogeneous among different cell types, with varying biophysical and pharmacological profiles, and as shown in this and the following examples can result from expression of different  $\alpha_1$  subunit subtypes in different cells.

**A. Cloning of  $\alpha_{1H-2}$**

As described above, PCR Primers-1 and -2, chosen based on an alignment of the human  $\alpha_{1A}$ - $\alpha_{1E}$  sequences in the central cytoplasmic loop II/III region and Primer-3 (GA(A/G)ATGATGATGAA(A/G)GT SEQ ID NO. 10) was chosen after considering  $\alpha_1$ -related *C. elegans* sequences in cosmid C54D2 aligned with the human  $\alpha_1$ -encoding nucleic acid sequences.

The  $\alpha_1$ -related encoding nucleic acids were amplified in two steps from TT cellular poly(A) + RNA, using Primers-1 and -2 first in a

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including the entire carboxy terminus and the in-frame translational stop codon.

DNA encoding the 5'-end of the human  $\alpha_{1H}$  calcium channel subunit was isolated using a 548 bp EcoRI-NcoI restriction endonuclease fragment from the 5'-end of clone 1.205 (nt 2432 to nt 2979 SEQ ID No. 49) to rescreen the TT cell cDNA library under high stringency conditions. Briefly, DNA encoding the amino terminus of human  $\alpha_{1H}$  calcium containing inserts of >3.5 kb was incubated with the purified restriction fragment and hybridized at 42 °C and washed under high stringency conditions as described above.

One recombinant, clone 1.305, was identified that contains a 3,530 nucleotide insert that shares at its 3' end approximately 1.1 kb of sequence identity with the 5'-end of clone 1.205 (~nt 2432 to nt 3530 SEQ ID No. 49) and also contains 2.4 kb of sequence upstream of the EcoRI site located at the 5'-end of clone 1.205 (nt 2433 to 2438 SEQ ID No. 49). This sequence encodes the ATG initiation codon (nt 249 to nt 251 SEQ ID No. 12) and 1,094 amino acids of the amino terminus of the  $\alpha_{1H}$  subunit as well as 248 bp of 5'-untranslated sequence, including a consensus ribosome binding site (nt 244 to nt 248 of SEQ ID No. 49).

Two other recombinants were also identified (SEQ ID NOs. 13 and 14) that share approximately 1.1 kb of sequence identity with the 3'-end of clone 1.305 but differ in the length of the DNA sequence corresponding to the extended intracellular loop located between transmembrane Domains I and II.

### 3. Construction of a full-length $\alpha_{1H-1}$ -encoding DNA clone

Portions of these partial cDNA clones can be ligated to generate a full-length  $\alpha_{1H}$  cDNA using common restriction endonuclease sites shared amongst the  $\alpha_{1H}$ -encoding fragments. A full-length  $\alpha_{1H}$  encoding clone was constructed by 1) combining the DNA encoding the 5'-end of  $\alpha_{1H}$  present

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Restriction endonuclease digestion of purified DNA isolated from  $\lambda$ 1.201- $\lambda$ 1.205 with EcoRI indicated that clone 1.201 contains the original insert of  $\sim 350$  bp PCR1 fragment, whereas clones 1.202, 1.203, 1.204 and 1.205 contain inserts of  $\sim 1100$ ,  $\sim 4000$ ,  $\sim 2600$  and  $\sim 2200$  nt, respectively.

**F. Isolation of DNA encoding a human  $\alpha_{1H}$  calcium channel subunit and construction of DNA encoding a full-length  $\alpha_{1H}$  subunit**

**1. Reference list of partial human  $\alpha_{1H}$  clones**

The full-length  $\alpha_{1H}$  cDNA sequence is set forth in SEQ ID NO. 49. A list of partial cDNA clones used to characterize the  $\alpha_{1H}$  sequence and the nucleotide position of each clone relative to the full-length  $\alpha_{1H}$  cDNA sequence is shown below. The isolation and characterization of these clones are described below.

	1.305	nt 1 to 3530 of SEQ ID No. 49
15	1.205	nt 2432 to 4658 of SEQ ID No. 49
	1.204	nt 3154 to 5699 of SEQ ID NO. 49
	PCR1	nt 4271 to 4610 of SEQ ID NO. 49
	1.202	nt 4372 to 5476 of SEQ ID No. 49
	1.203	nt 3891 to 7898 of SEQ ID No. 49

**20 2. Characterization of the clones**

DNA sequencing of each insert revealed that clone 1.202 contains 1,105 bp insert corresponding to nt 4372 to 5476 of SEQ ID No. 49; clone 1.203 contains 4,008 bp insert corresponding to nt 3891 to 7898 of SEQ ID No. 49; clone 1.204 contains 2,546 bp insert corresponding to nt 3154 to 5699 of SEQ ID NO. 49; and clone 1.205 contains 2,227 bp insert corresponding to nt 2432 to 4658 of SEQ ID No. 49. These four DNA clones contain overlapping sequences that encode an open reading frame of approximately 6.6 kb that encodes a majority of the  $\alpha_{1H}$  subunit,

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**F. Isolation and characterization of individual clones****Hybridization and Washing Conditions**

Hybridization of radiolabelled nucleic acids to immobilized DNA for the purpose of screening cDNA libraries, DNA Southern transfers, or northern transfers was routinely performed in standard hybridization conditions (hybridization: 50% deionized formamide, 200 µg/ml sonicated herring sperm DNA (Cat #223646, Boehringer Mannheim Biochemicals, Indianapolis, IN), 5 x SSPE, 5 x Denhardt's, 42° C.; wash :0.2 x SSPE, 0.1% SDS, 65° C). The recipes for SSPE and Denhardt's and the preparation of deionized formamide are described, for example, in Sambrook *et al.* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 8). In some hybridizations, lower stringency conditions were used in that 10% deionized formamide replaced 50% deionized formamide described for the standard hybridization conditions.

The washing conditions for removing the non-specific probe from the filters was either high, medium, or low stringency as described below:

- 1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C
- 2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C
- 3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C.

It is understood that equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

Approximately  $1.5 \times 10^5$  recombinants of the TT cell phage library containing inserts > 3.5 kb were plated and duplicate lifts prepared from each plate. The lifts were probed with radiolabelled PCR1 using standard hybridization conditions, the filters were washed and approximately 100 positive plaques were identified. Initially, 5 positives, A1.201-A1.205, were selected for plaque purification and characterization.

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available thermocycler (Perkin-Elmer) and the amplification reactions were set as follows: 1 cycle, 5 min @ 95 °C; 5 cycles, 20 sec @ 95 °C/20 sec @ 42 °C/2.5 min @ 72 °C; 30 cycles, 20 sec @ 95 °C/20 sec @ 50 °C/2.5 min @ 72 °C; and 1 cycle, 7 min @ 72 °C. Amplified DNA

5 products were subjected to electrophoresis on an agarose gel and gel purified using standard methods.

**E. Amplification of DNA encoding a portion of human  $\alpha_{1H}$  calcium channel subunit**

To amplify DNA encoding a portion of the human  $\alpha_{1H}$  calcium  
10 channel subunit, three degenerate oligonucleotides (SEQ ID NOs. 8-10) that share partial complementarity to a region of Domain III were synthesized as 5' primers. This region is encompassed within all of the amplified  $\alpha_1$ -encoding fragments of Section C above. Two  
15 oligonucleotides based on sequences in IIIS2 (SEQ ID NOs. 8 and 10) were used as 5' primers in conjunction with the 3'IIIS5 transmembrane primers used in the initial PCR reactions (SEQ ID NOs. 6 and 7 to amplify DNA encoding a portion of the human  $\alpha_{1H}$  subunit using the amplified products as templates.

The amplified DNA products were subcloned into the pCR-Blunt  
20 vector (Invitrogen), plasmid DNA was purified from isolated transformants and the DNA sequence of each insert was determined. A 340 bp fragment (SEQ ID NO. 48; nt 4271 to 4610 of SEQ ID NO. 49) that shares approximately 55-60% sequence identity to known human  $\alpha_1$  calcium channel subunits was identified. This DNA fragment, designated  
25 PCR1, was used as a DNA probe to isolate DNA encoding a human  $\alpha_{1H}$  calcium channels subunit.

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unligated adapters by column chromatography using Sepharose CL-4B resin followed by size selection of the cDNA on a 1.2% agarose gel. After visualizing the resolved DNA using ethidium bromide, two fractions of cDNA, >3.5 kb and 1.0-3.5 kb, were isolated from the gel and  
5 inserted into the vector  $\lambda$ gt10.

The ligated  $\lambda$ gt10 containing the cDNA insert was packaged into  $\lambda$  phage virions *in vitro* using the Gigapack III Gold packaging (Stratagene, La Jolla, CA) kit. Using this method, phage libraries of  $\sim 1.5 \times 10^6$  recombinants for cDNA >3.5 kb fraction and  $\sim 10 \times 10^6$  recombinants  
10 for DNA fraction between 1.0 and 3.5 kB were obtained.

**D. Isolation of DNA encoding a portion of human  $\alpha_1$  calcium channel subunits**

DNA encoding a small region of human  $\alpha_1$  subunits encoded in TT cells was isolated using degenerate PCR-based amplification (e.g., see  
15 Williams *et al.* (1994) J. Biol. Chem. 269:22347-22357). These amplified fragments were used to generate DNA probes for the isolation of DNA encoding a full-length human  $\alpha_{1H}$  calcium channel subunit.

As noted above, two sets of degenerate oligonucleotides were synthesized based on the flanking regions of the II-III loop known to share  
20 a high degree of sequence identity amongst known human  $\alpha_1$  calcium channel subunits: 1) two degenerate oligonucleotides complementary to the regions of the IIS5-IIS6 loop were synthesized as 5' upstream primers (SEQ ID NOs. 4 and 5); and 2) two degenerate oligonucleotides complementary to a portion of the IIS5 transmembrane segment were  
25 synthesized as 3' downstream primers (SEQ ID NOs. 6 and 7).

These degenerate oligonucleotides were used as primer pairs in nested PCR amplification reactions using Pfu DNA polymerase (Stratagene, La Jolla, CA) and reactions were performed according to the manufacturer's instructions. Samples were placed in a commercially

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PolyA + RNA was isolated from total cytoplasmic RNA using two passes over an oligo(dT)-cellulose column. Briefly, 2-3 mg of total cytoplasmic RNA was resuspended in NETS buffer (500 mM NaCl 10 mM EDTA, 10 mM Tris, pH 7.4, 0.2% SDS) and passed slowly over a column containing 0.5 g of oligo(dT)-cellulose (Collaborative Research) equilibrated in NETS buffer. The column was washed with 30 mls of NETS buffer and polyA + RNA was eluted using about 3 mls of ETS buffer (10 mM EDTA, 10 mM Tris, pH 7.4, 0.2% SDS). The ionic strength of the polyA + RNA-containing buffer was adjusted to 500 mM NaCl and passed over a second oligo(dT)-cellulose column essentially as described above. Following elution from the second column, the polyA + RNA was precipitated twice in ethanol and resuspended in H<sub>2</sub>O.

#### C. Library construction

Double stranded cDNA (dscDNA) was synthesized according to standard methods (see, *e.g.*, Gubler *et al.* (1985) Gene 25:263-269; Lapeyre *et al.* (1985) Gene 37:215-220). Briefly, first strand cDNA synthesis was initiated using TT cell polyA + RNA as a template and using random primers and Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT). The second strand was synthesized using a combination of E. coli DNA polymerase, E. coli DNA ligase and RNase H.

Regions of single stranded DNA were converted to double-stranded DNA using T4 DNA polymerase generating blunt-ended double stranded fragments. EcoRI restriction endonuclease site adapters:

5' CGTGACGTCACGCTAG 3' (SEQ ID NO. 2)  
25 3' GCACGTGCAGTGCGATCTTAA 5' (SEQ ID NO. 3)

were ligated to the double-stranded cDNA using a standard protocol (see, *e.g.*, Sambrook *et al.* (1989) IN: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 8). The double-stranded DNA with the *EcoRI* adapters ligated was purified away from the free or



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**Isolation and Characterization of the clones:** A recombinant cDNA library was constructed in phage vector  $\lambda$ gt10 using poly(A)<sup>+</sup>-selected RNA from the TT cell line. Approximately  $1.5 \times 10^6$  were screened with the PCR fragment under high stringency (hybridization: 50% formamide, 5X SSPE, 5X Denhardts, 0.2% SDS, 200 $\mu$ g/ml herring sperm DNA for 16-18 hrs. at 42°C; wash: 6 washes of 30 minutes each in 0.1X SSPE, 0.1% SDS at 65°C).

**Northern blot analysis:** Multiple tissues were screened in Northern blots using 2 $\mu$ g of poly(A)<sup>+</sup> RNA per lane (Clontech, Palo Alto, CA). Blots were probed at high stringency, as described above, with labeled fragments generated from the full-length  $\alpha_{1H}$  cDNA, i.e., nucleotide -6 to 7390.

**Western blot analysis:** Cellular membranes (total) were isolated from HEK293 cells expressing different  $\alpha_{1H}$  subunits; membrane proteins were separated by SDS-PAGE; transferred to nitrocellulose; and, blotted using a polyclonal anti- $\alpha_{1H}$  antisera and TBS-T buffer. Blotted proteins were visualized using the Lumiglo reagent kit (KPL, Gaithersburg, MD) according to the manufacturer's instructions.

#### **B. RNA isolation**

Human medullary thyroid carcinoma cells (TT cells; ATCC Accession No. CRL1803) were grown in DMEM medium supplemented with 10 % fetal calf serum at 37 °C in 5% CO<sub>2</sub> atmosphere and total cytoplasmic RNA was isolated from forty 10 cm plates using a "midi-prep" RNA isolation kit (Qiagen) as per the manufacturer's instructions. The protocol entails the use of the detergent NP40 which lyses the cell membrane under mild conditions such that the nuclear membrane remains intact thereby eliminating incompletely spliced RNA transcripts from the preparation.

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**A. Materials and Methods****Nucleic acid amplification:**

The following sense strand 20-mer PCR primer, corresponding to nucleotides 1945-1964 of DNA encoding a human  $\alpha_{1E}$  subunit, was

5 synthesized:

AC(A/C/G/T)GTGTT(C/T)CAGATCCTGAC (Primer-1) SEQ ID NO. 4

An antisense 22-nucleotide PCR primer, corresponding to nucleotides 3919 through 3940 of human  $\alpha_{1E}$ , was also synthesized:

T(C/T)CCCTTGAAGAGCTG(A/C/G/T)ACCCC (Primer-2) SEQ ID NO. 1

10 The sense and the antisense primers were used in amplification reactions with cDNA prepared from TT cells and Pfu DNA polymerase (Stratagene Inc., San Diego, CA).

Reaction conditions: 95°C for 5 minutes followed by 5 cycles of 20 seconds each at 95°C; then 20 seconds at 42°C; 2.5 minutes at 15 72°C; and, 30 cycles of 20 seconds each at 95°C followed by 20 seconds at 50°C and finally 2.5 minutes at 72°C. The product of the reaction is referred to herein (below) as "the original PCR products."

A second 5' degenerate oligonucleotide primer was designed corresponding to a portion of the sequence reported for *C. elegans*, 20 cosmid C54D2 (Genebank accession #U37548), as a portion of that sense strand sequence which aligns with a portion of the human  $\alpha_{1E}$  subunit DNA sequence between nucleotide 3598 and 3614. This primer had the following sequence:

GA(A/G)ATGATGATGAA(A/G)GT (Primer-3) SEQ ID NO. 10

25 Primer-3 was used in a nested amplification reaction with the original PCR products and the Primer-2.

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- identifying compounds useful in treating: (a) disorders of sexual function including impotence; and (b) alcoholic impotence (under autonomic control that may be subject to T-channel controls); hepatic, for identifying compounds useful in treating and reducing neuronal toxicity and
- 5 autonomic nervous system damage resulting from acute over-consumption of alcohol; neurological conditions for identifying compounds useful in treating: (a) epilepsy and diencephalic epilepsy; (b) Parkinson disease; (c) aberrant temperature control, such as abnormalities of shivering and sweat gland secretion and peripheral
- 10 vascular blood supply; (d) aberrant pituitary and hypothalamic functions including abnormal secretion of noradrenaline, dopamine and other hormones; respiratory conditions, for identifying compounds useful in treating abnormal respiration, such as, post-surgical complications of anesthetics; endocrine
- 15 disorders for identifying compounds useful in treating aberrant secretion of hormones such as treatments for overproduction of hormones including insulin, thyroxin, and adrenalin.

#### EXAMPLES

- The following examples are included for illustrative purposes only
- 20 and are not intended to limit the scope of the invention.

##### EXAMPLE 1: ISOLATION OF DNA ENCODING THE HUMAN CALCIUM CHANNEL $\alpha_{1H-1}$ SUBUNIT

- Using mRNA and TT cells, a degenerate PCR approach was used to isolate nucleic acid encoding an  $\alpha_1$  subunit. Nucleic acid encoding an  $\alpha_{1H-1}$
- 25 subunit and nucleic acid encoding a subunit designated as  $\alpha_{1H-2}$  was isolated. The nucleic acid was introduced into HEK293 cells and *Xenopus* oocytes and voltage gated calcium channels were expressed. These channels exhibit pharmacological and electrophysiological properties consistent with native LVA, T-type, channels.

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can be screened to determine if they are also predisposed to such disease states.

Disorders and for which screening assays can be developed and also for which candidate compounds for treatment of the disorders

5 include, but are not limited to: cardiac treatments, such as myocardial infarct, cardiac arrhythmia, heart failure, and angina pectoris. Identified compounds will be useful in: (a) adjunctive therapies for reestablishing normal heart rate and cardiac output following traumatic injury, heart attack and other heart injuries; (b) treatments of myocardial infarct (MI),

10 post-MI and in an acute setting. The compounds may be effective to increase cardiac contractile force, such as that measured by left ventricular enddiastolic pressure, and without changing blood pressure or heart rate. In an acute setting the compounds may be effective to decrease formation of scar tissue, such as that measured by collagen

15 deposition or septal thickness, and without cardiodepressant effects. The identified compounds will be useful for and assays for diagnosis and compound screening will be useful in connection with vascular treatments and hypertension, for identifying compounds useful in regulating vascular smooth muscle tone, including vasodilating or

20 vasoconstricting. Such compounds can be used in (a) treatments for reestablishing blood pressure control, e.g., following traumatic injury, surgery or cardiopulmonary bypass, and in prophylactic treatments designed to minimizing cardiovascular effects of anaesthetic drugs; (b) treatments for improving vascular reflexes and blood pressure control by

25 the autonomic nervous system. Other conditions include urologic, for identifying compounds useful in: (a) treating and restoring renal function following surgery, traumatic injury, uremia and adverse drug reactions; (b) treating bladder dysfunctions; and (c) uremic neuronal toxicity and hypotension in patients on hemodialysis; reproductive conditions, for

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### Pharmacological properties

The pharmacological properties of  $\alpha_{1H}$ -containing calcium channels were also consistent with those observed for native T-type calcium channels. Interestingly, the sensitivity of  $\alpha_{1H.1}$ -containing calcium channels to  $Cd^{2+}$  or Amiloride was about 10-fold lower when expressed in HEK293 cells than when expressed in *Xenopus* oocytes.

The data indicate that human  $\alpha_{1H}$  calcium channel subunits have properties consistent with that of native T-type calcium channels and, as such,  $\alpha_{1H}$  represent a member in the rapidly growing family of low-voltage activated calcium channels.

### Assays for diagnosis of LVA-calcium channel mediated disorders and clinical applications

#### Clinical applications

In relation to therapeutic treatment of various disease states, the availability of DNA encoding human calcium channel subunits permits identification of any alterations in such genes (e.g., mutations) which may correlate with the occurrence of certain disease states. In addition, the creation of animal models of such disease states becomes possible, by specifically introducing such mutations into synthetic DNA fragments that can then be introduced into laboratory animals or *in vitro* assay systems to determine the effects thereof.

Also, genetic screening can be carried out using the nucleotide sequences as probes. Thus, nucleic acid samples from subjects having pathological conditions suspected of involving alteration/modification of any one or more of the calcium channel subunits can be screened with appropriate probes to determine if any abnormalities exist with respect to any of the endogenous calcium channels. Similarly, subjects having a family history of disease states related to calcium channel dysfunction

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the steady-state inactivation properties, were similar to those obtained in HEK293 cells (see EXAMPLES).

Single-channel properties of  $\alpha_{1H}Ca^{2+}$  channels in HEK293 cells were determined in cell-attached recordings with 110 mM  $Ba^{2+}$  as the charge carrier. Single-channel recordings at a test potential of -30 mV from a patch that contains at least three  $\alpha_{1H}$  showed that channel openings occurred in bursts and were clustered mainly in the first third of the 100-ms depolarizing pulse, especially with stronger depolarizations. Occasionally, channel activity was spread throughout the entire sweep.

10 The time course of the ensemble-averaged current recorded at -30mV in 110 mM  $Ba^{2+}$  was similar to the  $\alpha_{1H}$  whole-cell  $Ba^{2+}$  current recorded at -40 mV in 15 mM  $Ba^{2+}$ . The currents were compared at different potentials to compensate for the shift in the activation curve to more positive potentials due to the increase in divalent concentration. The

15 unitary current-voltage relationship yielded a unitary slope conductance of  $9.06 \pm 0.22$  pS ( $n=4$ ).

#### Summary of Electrophysiologic Characteristics

The biophysical properties of calcium channels containing the human  $\alpha_{1H}$  subunit were evaluated. Whole cell recordings from transiently

20 transfected HEK293 cells indicate that the current-voltage relationship, permeability to  $Ca^{2+}$  and  $Ba^{2+}$ , kinetics of activation, and single channel conductance of calcium channels containing  $\alpha_{1H}$  subunits were similar to those of native T-type calcium channels in tissues. Tail currents from  $A_{1H}$  channels showed a bi-exponential decay, exhibiting a fast and a slower

25 component. At very negative membrane potentials (-150 to -100 mV) the fast component ( $\tau$ : 200-450  $\mu$ s) dominated the inactivation process, while at depolarizing potentials  $>-50$  mV the slower component (2-3 ms) dominated. At the resting membrane potential, i.e.,  $\leq -80$  mV, both components contribute equally.

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two Boltzmann functions (Figure 1). The potentials for half-maximal activation of the individual Boltzmann terms were as follows:  $V_{1/2,A}$ :  $-25.1 \pm 3.3$  mV; and  $V_{1/2,B}$ :  $+25.5 \pm 3.9$  mV ( $n = 11$ ). A value similar to  $V_{1/2,A}$  has been reported previously for voltage dependence of activation of T-type  $Ca^{2+}$  channels in the human TT cell line ( $-27$  mV). The value of the second Boltzmann term  $V_{1/2,B}$  is somewhat similar to that reported for HVA  $Ca^{2+}$  channels. Using a similar protocol, tail currents of HVA  $Ca^{2+}$  channels decay with time constants of  $< 300 \mu s$ , whereas with  $\alpha_{1H}$  the most prominent at test potentials close to  $V_{1/2,B}$ . The availability of  $\alpha_{1H}$  containing  $Ca^{2+}$  channels for opening was dependent on the membrane potential as shown in Fig. 1. The potential for half-maximal steady-state inactivation ( $V_{1/2}$ ) was  $-63.2 \pm 2.0$  mV ( $n = 9$ ).

The rapid inactivation of  $\alpha_{1H}$   $Ca^{2+}$  channels was strongly voltage-dependent. The current decay was best described with an exponential function with time constants ranging from  $42.2 \pm 7.8$  to  $8.8 \pm 3.8$  ms at membrane potentials between  $-50$  and  $+30$  mV ( $n = 6$ ; data not shown). Activation kinetics of  $\alpha_{1H}$   $Ca^{2+}$  channels were also voltage-dependent with time constants ranging from  $9.9 \pm 4.7$  to  $0.9 \pm 0.3$  ms for membrane potentials between  $-50$  and  $+30$  mV ( $n = 8$ ; data not shown).  $\alpha_{1H}$   $Ca^{2+}$  channels inactivated completely during the 150-ms depolarization. Recovery from inactivation occurred within a period of  $\sim 3$  s with a fast component ( $\tau = 37 \pm 9$  ms;  $16.5 \pm 4.6\%$  of all channels) and a slow component ( $\tau = 37 \pm 61$  ms;  $78 \pm 8.5\%$  of all channels;  $n = 3$ ; data not shown). To confirm the biophysical properties of recombinant  $\alpha_{1H}$  channels observed in whole-cell recordings from HEK293 cells, the functional expression of  $\alpha_{1H}$  in *Xenopus* oocytes was tested. Substantial currents ( $< 1 \mu A$ ) after injection of  $\alpha_{1H}$  transcripts alone was observed. The activation and inactivation kinetics, as well as

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depolarizations to various test potentials from a holding potential of -90 mV were measured. Currents were activated at a test potential of -50 mV, peaked between -20 and -10 mV, and reversed at a membrane potential more positive than +60 mV. Similar results were obtained with

5  $\text{Ca}^{2+}$  (15 mM) as the charge carrier.

One hallmark of LVA channels is their slow rate of deactivation, which is reflected in a slow decay of tail currents. The time constant of this decay is ~10-fold slower for LVA channels (2-12 ms) than for HVA channels  $< 300 \mu\text{s}$ . A slow decay of  $\alpha_{1H-1}$  mediated tail currents over a

10 period of ~15 ms was observed. In contrast to the monoexponential decay of the tail currents reported for many native T-type  $\text{Ca}^{2+}$  channels, tail currents from  $\alpha_{1H-1}$  channels showed a biexponential decay. At a test potential of -20 mV, the decay rate of the slow component, comprising  $88.1 \pm 33.8\%$  of the total current, was  $2.1 \pm 1.06 \text{ ms}$  ( $n=6$ ), which is

15 similar to those observed in native T-type  $\text{Ca}^{2+}$  channels. The decay rate of the faster component was  $0.64 \pm 0.21 \text{ ms}$  ( $n = 6$ ).

Whole-cell patch clamp recordings were performed on HEK293 cells transiently expressing the human  $\alpha_{1H-1}$  subunit. Step-depolarizations elicited inward  $\text{Ba}^{2+}$  currents that activate slowly and inactivate rapidly

20 ( $2.8 \pm 0.6$  and  $16.9 \pm 5.3 \text{ ms}$ , at -20 mV). The activation curve of  $\alpha_{1H-1}$  is shifted to the left ( $V_{1/2}:-29.5 \text{ mV}$ ) compared to HVA  $\text{Ca}^{2+}$  channels. The tail currents of  $\alpha_{1H-1}$ -containing channels decay slowly ( $\tau_1, \tau_2 \pm 1.0, 0.6, \pm 0.2 \text{ ms}$ ). The permeability for  $\text{Ba}^{2+}$  and  $\text{Ca}^{2+}$  was virtually identical. The single channel conductance, determined with 110 mM  $\text{Ba}^{2+}$

25 as charge carrier, is 9pS.

The voltage dependence of activation of  $\alpha_{1H-1}$  containing  $\text{Ca}^{2+}$  channels was determined from tail-current analysis. Normalized tail-current amplitudes were plotted as a function of test potential and revealed a biphasic activation curve that was well fitted by the sum of



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these two different subunits should be useful for targeting therapeutic intervention into selected neural locations, e.g., to cardiovascular neurons an cardiac pacemaker neurons expressing  $\alpha_{1H-2}$ . Calcium channels formed from  $\alpha_{1H}$  subunits open at small changes in membrane potential, but only  
 5 allow moderate  $Ca^{2+}$  influx before closing. By allowing moderate influx of divalent ions the  $\alpha_{1H}$  containing channels are likely to:

- (i) participate in pathways triggering changes in gene expression in response to subtle change sin membrane potential difference, i.e., in neuronal and non-neuronal cell types (e.g., in activation  
 10 of immune cells such as T-cells, in activation of kidney and liver cells in response to metabolic changes;
- (ii) exert subtle controls over the overall excitability or accessibility of neurons to synaptic transmission, such as in determining which neurons will respond to stimulae, and to what extent, such as in  
 15 peripheral neurons and ganglia;
- (iii) determine the extent of neural responses to stimulae such as chronic pain;
- (iv) regulate the sensitivity of neurons in critical neural centers so that neuronal cells in these centers are protected from the adverse effects  
 20 associated with excessive bursts of firing (e.g., in the cardiac pacemaker);
- (v) act to set the steady state pattern of inactivation of neurons in different regions of the brain, (e.g., in response to sleep, sex, emotion, depression, fatigue and the other stimulae or conditions).

25 **Electrophysiology of cells that express channels containing the  $\alpha_{1H-1}$  subunit**

#### **Expression of recombinant $\alpha_{1H-1}$ channels**

Following transient transfection of HEK293 cells with a DNA encoding the  $\alpha_{1H}$  subunit,  $Ba^{2+}$  currents that were rapidly activating and inactivating were observed.  $Ba^{2+}$  currents (15 mM) elicited by step

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receptors to cellular regulation, particularly since the observed region of sequence divergence resides in the cytosolic linker region between domains I and II and the analogous sequence region in high-voltage activated calcium channels has been implicated in binding of cytosolic regulatory proteins. Observed differences in biophysical properties of  $\alpha_{1H-1}$  and  $\alpha_{1H-2}$  are also likely indicative of differences in the sensitivity of these two different channel subunits to pharmaceutical compounds. Thus, it seems likely that low-voltage activated calcium channels containing either the  $\alpha_{1H-1}$  or the  $\alpha_{1H-2}$  subunit will be subject to different regulatory controls, and different profiles of susceptibility to pharmaceutical compounds. For example, amiloride blocks the T-type current in neuroblastoma cells with an  $IC_{50}$  of  $\sim 50 \mu M$ , whereas in hippocampal neurons  $300 \mu M$  amiloride reduces the T-type current by only 40%.

15 In this respect, each a different  $\alpha_{1H}$  channel is a separate screening target for development of pharmaceutical drug compounds. Differential effects of drugs on different neural cells and in different neural tissues can be understood based on different patterns of expression of  $\alpha_{1H-1}$  and/or  $\alpha_{1H-2}$  *in vivo* and will provide a means to identify drugs specific for each subtype and associated disorders or conditions. The observed sequence variation in  $\alpha_{1H}$  subunits explains observed pharmacological variability of T-type calcium channels in different native tissues, providing a useful tool to identify where the respective  $\alpha_{1H-1}$  and  $\alpha_{1H-2}$  subunit is expressed to use screening assays to identify targeted therapeutic drug candidates.

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Differences in  $\alpha_{1H-1}$  and  $\alpha_{1H-2}$  functionality and expression in different tissues provides basis for using recombinant cells expressing calcium channels having either the  $\alpha_{1H-1}$  or  $\alpha_{1H-2}$  subunit. Agonists and antagonists capable of differentially affecting calcium channels containing

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reaction which occurs intracellularly and which is directly influenced in a calcium (or other) ion dependent manner. Any method for assessing the activity of a calcium channel may be used in conjunction with the cells and assays provided herein. For example, in one embodiment of

5 the method for testing a compound for its ability to modulate calcium channel activity, the amount of current is measured by its modulation of a reaction which is sensitive to calcium channel-selective ions and uses a eukaryotic cell which expresses a heterologous calcium channel and also contains a transcriptional control element operatively linked for expression

10 to a structural gene that encodes an indicator protein. The transcriptional control element used for transcription of the indicator gene is responsive in the cell to a calcium channel-selective ion, such as  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$ . The details of such transcriptional based assays are described in commonly owned PCT International Patent Application No. PCT/US91/5625, filed

15 August 7, 1991, which claims priority to copending commonly owned allowed U.S. Application Serial No. 07/563,751, filed August 7, 1990; see also, commonly owned published PCT International Patent Application PCT US92/11090, which corresponds to co-pending U.S. Applications Serial Nos. 08/229,150 and 08/244,985. The contents of these

20 applications are herein incorporated by reference thereto.

#### **Biophysical and pharmacological properties of $\alpha_{1H}$ subunits**

HEK cells were transfected with DNA and oocytes injected with nucleic acid provided herein. The cell expressed calcium channels, which were then characterized electrophysiologically and pharmacologically.

25 These results are described in the examples. Both splice variants formed calcium channels that exhibit properties associated with T-type channels. Variant-specific properties were observed.

These observed differences in the amino acid sequences of  $\alpha_{1H.1}$  and  $\alpha_{1H.2}$  will result in marked differences in susceptibility of these

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- desirably may be used in assays to identify agents, such as calcium channel agonists and antagonists, that modulate calcium channel activity. Functionally testing the activity of test compounds, including compounds having unknown activity, for calcium channel agonist or antagonist
- 5 activity to determine if the test compound potentiates, inhibits or otherwise alters the flow of calcium ions or other ions through a human calcium channel can be accomplished by (a) maintaining a eukaryotic cell which is transfected or injected to express a heterologous functional calcium channel capable of regulating the flow of calcium channel-
- 10 selective ions into the cell in a medium containing calcium channel-selective ions (i) in the presence of and (ii) in the absence of a test compound; (b) maintaining the cell under conditions such that the heterologous calcium channels are substantially closed and endogenous calcium channels of the cell are substantially inhibited (c) depolarizing the
- 15 membrane of the cell maintained in step (b) to an extent and for an amount of time sufficient to cause (preferably, substantially only) the heterologous calcium channels to become permeable to the calcium channel-selective ions; and (d) comparing the amount and duration of current flow into the cell in the presence of the test compound to that of
- 20 the current flow into the cell, or a substantially similar cell, in the absence of the test compound.

The assays thus use cells, provided herein, that express heterologous functional calcium channels and measure functionally, such as electrophysiologically, the ability of a test compound to potentiate,

25 antagonize or otherwise modulate the magnitude and duration of the flow of calcium channel-selective ions, such as  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$ , through the heterologous functional channel. The amount of current which flows through the recombinant calcium channels of a cell may be determined directly, such as electrophysiologically, or by monitoring an independent

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similar studies which have been carried out with the acetylcholine receptor, see, *e.g.*, Claudio *et al.* ((1987) *Science* 238:1688-1694).

Transcription based assays are also contemplated herein.

- Functional recombinant or heterologous calcium channels may be
- 5 identified by any method known to those of skill in the art. For example, electrophysiological procedures for measuring the current across an ion-selective membrane of a cell, which are well known, may be used. The amount and duration of the flow of calcium-selective ions through heterologous calcium channels of a recombinant cell containing DNA
- 10 encoding one or more of the subunits provided herein has been measured using electrophysiological recordings using a two electrode and the whole-cell patch clamp techniques. In order to improve the sensitivity of the assays, known methods can be used to eliminate or reduce non-calcium currents and calcium currents resulting from endogenous calcium
- 15 channels, when measuring calcium currents through recombinant channels. For example, the DHP Bay K 8644 specifically enhances L-type calcium channel function by increasing the duration of the open state of the channels (see, *e.g.*, Hess, J.B., *et al.* (1984) *Nature* 311:538-544). Prolonged opening of the channels results in calcium currents of increased
- 20 magnitude and duration. Tail currents can be observed upon repolarization of the cell membrane after activation of ion channels by a depolarizing voltage command. The opened channels require a finite time to close or "deactivate" upon repolarization, and the current that flows through the channels during this period is referred to as a tail current.
- 25 Because Bay K 8644 prolongs opening events in calcium channels, it tends to prolong these tail currents and make them more pronounced.

In practicing these assays, stably or transiently transfected cells or injected cells that express voltage-dependent human calcium channels containing one or more of the subunits of a human calcium channel

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specifically bind to the membrane or alter or modulate the activity of a heterologous calcium channel on the membrane.

In preferred embodiments, the assay uses a recombinant cell that has a calcium channel containing an  $\alpha_1$  subunit of a human calcium  
5 channel. In other preferred embodiments, the assay uses a recombinant cell that has a calcium channel containing an  $\alpha_1$  subunit of a human calcium channel in combination with a  $\beta$  subunit of a human calcium channel and/or an  $\alpha_2$  subunit of a human calcium channel. Recombinant  
10 cells expressing heterologous calcium channels containing each of the  $\alpha_1$  and optionally a  $\beta$  and/or  $\alpha_2$  human subunits, and, optionally, a  $\gamma$  subunit of a human calcium channel are especially preferred for use in such assays.

In certain embodiments, the assays for identifying compounds that modulate calcium channel activity are practiced by measuring the calcium  
15 channel activity of a eukaryotic cell having a heterologous, functional calcium channel when such cell is exposed to a solution containing the test compound and a calcium channel-selective ion and comparing the measured calcium channel activity to the calcium channel activity of the same cell or a substantially identical control cell in a solution not  
20 containing the test compound. The cell is maintained in a solution having a concentration of calcium channel-selective ions sufficient to provide an inward current when the channels open. Recombinant cells expressing calcium channels that include each of the  $\alpha_1$ ,  $\beta$  and  $\alpha_2$  human subunits, and, optionally, a  $\gamma$  subunit of a human calcium channel, are especially  
25 preferred for use in such assays. Methods for practicing such assays are known to those of skill in the art. For example, for similar methods applied with *Xenopus laevis* oocytes and acetylcholine receptors, see, Mishina *et al.* ((1985) *Nature* 313:364) and, with such oocytes and sodium channels (see, Noda *et al.* (1986) *Nature* 322:826-828). For

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channels that contain one or all of the human calcium channel subunits provided herein.

With respect to ligand binding assays for identifying a compound which has affinity for calcium channels, cells are employed which

5 express, preferably, at least a heterologous  $\alpha_1$  subunit. Transfected eukaryotic cells which express at least an  $\alpha_1$  subunit may be used to determine the ability of a test compound to specifically bind to heterologous calcium channels by, for example, evaluating the ability of

10 the test compound to inhibit the interaction of a labeled compound known to specifically interact with calcium channels. Such ligand binding assays may be performed on intact transfected cells or membranes prepared therefrom.

The capacity of a test compound to bind to or otherwise interact with membranes that contain heterologous calcium channels or subunits

15 thereof, preferably  $\alpha_{1H}$  subunit-containing calcium channels, may be determined by using any appropriate method, such as competitive binding analysis, such as Scatchard plots, in which the binding capacity of such membranes is determined in the presence and absence of one or more concentrations of a compound having known affinity for the calcium

20 channel. Where necessary, the results may be compared to a control experiment designed in accordance with methods known to those of skill in the art. For example, as a negative control, the results may be compared to those of assays of an identically treated membrane preparation from host cells which have not been transfected with one or

25 more subunit-encoding nucleic acids.

The assays involve contacting the cell membrane of a recombinant eukaryotic cell which expresses at least one subunit of a human calcium channel, preferably at least an  $\alpha_1$  subunit of a human calcium channel, with a test compound and measuring the ability of the test compound to

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channels using eukaryotic cells that express heterologous human calcium channels are provided.

- In particular, the assays use eukaryotic cells that express homomeric or heteromeric human calcium channel subunits encoded by heterologous DNA provided herein, for screening potential calcium channel agonists and antagonists which are specific for human calcium channels and particularly for screening for compounds that are specific for particular human calcium channel subtypes. Such assays may be used in conjunction with methods of rational drug design to select among agonists and antagonists, which differ slightly in structure, those particularly useful for modulating the activity of human calcium channels, and to design or select compounds that exhibit subtype- or tissue-specific calcium channel antagonist and agonist activities. These assays should accurately predict the relative therapeutic efficacy of a compound for the treatment of certain disorders in humans. In addition, since subtype- and tissue-specific calcium channel subunits are provided, cells with tissue- specific or subtype-specific recombinant calcium channels may be prepared and used in assays for identification of human calcium channel tissue- or subtype-specific drugs.
- Desirably, the host cell for the expression of calcium channel subunits does not produce endogenous calcium channel subunits of the type or in an amount that substantially interferes with the detection of heterologous calcium channel subunits in ligand binding assays or detection of heterologous calcium channel function, such as generation of calcium current, in functional assays. Also, the host cells preferably should not produce endogenous calcium channels which detectably interact with compounds having, at physiological concentrations (generally nanomolar or picomolar concentrations), affinity for calcium



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LVA currents in a number of cell types. In contrast, ethosuximide, an antiepileptic agent that inhibits LVA currents in some cell types, had no effect on  $\alpha_{1H}$ -mediated currents. Although the L-type  $\text{Ca}^{2+}$ -channel modulators nimodipine and (-)-Bay K 8644 had little effect at a concentration of  $1\mu\text{M}$  on  $\alpha_{1H}$ -containing channels, both compounds produced a marked inhibition at a concentration of  $10\mu\text{M}$ , consistent with their effects on T-type channels in rat hypothalamic neurons (Akaike et al., 1989). In summary, the pharmacological properties of  $\alpha_{1H}$ -containing channels described here have many similarities to native T-type channels studied in a variety of cell types. The pharmacological profiles of T-type channels vary considerably between cell types, and no hallmark pharmacological feature of T-type channels has been identified. These results are consistent with the finding herein that multiple  $\alpha_1$  subunits are responsible for the pharmacological profiles of a family of LVA, or T-type, channels.

#### Assays and Clinical uses of the cells and calcium channels

##### Assays

##### Assays for identifying compounds that modulate calcium channel activity

Among the uses for eukaryotic cells which recombinantly express one or more subunits are assays for determining whether a test compound has calcium channel agonist or antagonist activity. These eukaryotic cells may also be used to select from among known calcium channel agonists and antagonists those exhibiting a particular calcium channel subtype specificity and to thereby select compounds that have potential as disease- or tissue-specific therapeutic agents.

*In vitro* methods for identifying compounds, such as calcium channel agonist and antagonists, that modulate the activity of calcium

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well known in the art. Transcripts of any of the full-length DNA encoding any of the subunits of a human calcium channel may be injected alone or in combination with other transcripts into eukaryotic cells for expression in the cells. Amphibian oocytes are particularly preferred for expression of *in vitro* transcripts of the human calcium channel subunit cDNA clones provided herein. Amphibian oocytes that express functional heterologous calcium channels have been produced by this method.

#### Pharmacological and electrophysiological properties

As described in the examples, nucleic acid encoding  $\alpha_{1H-1}$  and nucleic acid encoding  $\alpha_{1H-2}$  has been expressed in mammalian cells and in amphibian oocytes. Electrophysiological and pharmacological properties have been studied.

The biophysical properties of recombinant human  $\alpha_{1H}^{2+}$  channels expressed in HEK293 cells and *Xenopus* oocytes are in good agreement, indicating that the biophysical properties of recombinant human  $\alpha_{1H}$  channels are independent of the expression system. Several biophysical characteristics support the conclusion that the human  $\alpha_{1H}$  subunit is the pore-forming  $\alpha_1$  subunit of a T-type channel. The rates of activation, inactivation, and deactivation and the single-channel conductance of  $\alpha_{1H}$ -containing channels are within the ranges described for T-type channels. The conductance value of 9 pS measured in this study is near the value determined for rat  $\alpha_{1G}$ -containing channels and is significantly lower than those determined for recombinant HVA channels. In addition,  $\alpha_{1H}$ -containing channels conduct  $Ba^{2+}$  and  $Ca^{2+}$  equally well, consistent with the finding that the conductance of T-type channels for  $Ba^{2+}$  and  $Ca^{2+}$  is nearly equivalent in most cell types.

$\alpha_{1H}$ -containing  $Ca^{2+}$  channels display a pharmacological profile differing from those of HVA channels.  $\alpha_{1H}$ -mediated currents are inhibited by  $Ni^{2+}$ , amiloride, and mibefradil (Ro 40-5967), agents shown to reduce

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HEK cells that have been transiently transfected with DNA encoding  $\alpha_{1B-1}$ ,  $\alpha_{2b}$ , and  $\beta_{1-2}$  express heterologous calcium channels that exhibit sensitivity to  $\omega$ -conotoxin and currents typical of N-type channels. It has been found that alteration of the molar ratios of  $\alpha_{1B-1}$ ,  $\alpha_{2b}$  and  $\beta_{1-2}$  introduced into the cells to achieve equivalent mRNA levels significantly increased the number of receptors per cell, the current density, and affected the  $K_d$  for  $\omega$ -conotoxin.

The electrophysiological properties of these channels produced from  $\alpha_{1B-1}$ ,  $\alpha_{2b}$ , and  $\beta_{1-2}$  was compared with those of channels produced by transiently transfecting HEK cells with DNA encoding  $\alpha_{1B-1}$ ,  $\alpha_{2b}$  and  $\beta_{1-3}$ . The channels exhibited similar voltage dependence of activation, substantially identical voltage dependence, similar kinetics of activation and tail currents that could be fit by a single exponential. The voltage dependence of the kinetics of inactivation was significantly different at all voltages examined.

In certain embodiments, the eukaryotic cell with a heterologous calcium channel is produced by introducing into the cell a first composition, which contains at least one RNA transcript that is translated in the cell into a subunit of a human calcium channel. In preferred embodiments, the subunits that are translated include an  $\alpha_1$  subunit of a human calcium channel. More preferably, the composition that is introduced contains an RNA transcript which encodes an  $\alpha_1$  subunit of a human calcium channel and also contains (1) an RNA transcript which encodes a  $\beta$  subunit of a human calcium channel and/or (2) an RNA transcript which encodes an  $\alpha_2$  subunit of a human calcium channel. Especially preferred is the introduction of RNA encoding an  $\alpha_1$ , a  $\beta$  and an  $\alpha_2$  human calcium channel subunit, and, optionally, a  $\gamma$  subunit of a human calcium channel. Methods for *in vitro* transcription of a cloned DNA and injection of the resulting RNA into eukaryotic cells are

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or at least a human  $\alpha_1$  subunit and optionally an  $\alpha_2\delta$  subunit and/or a human  $\beta$  subunit are preferred. Eukaryotic cells transformed with a composition containing DNA or an RNA transcript that encodes an  $\alpha_1$  subunit alone or in combination with a  $\beta$  and/or an  $\alpha_2$  subunit may be used to produce cells that express functional calcium channels. Since recombinant cells expressing human calcium channels containing all of the human subunits encoded by the heterologous DNA or RNA are especially preferred, it is desirable to inject or transfect such host cells with a sufficient concentration of the subunit-encoding nucleic acids to form calcium channels that contain the human subunits encoded by heterologous DNA or RNA. The precise amounts and ratios of DNA or RNA encoding the subunits may be empirically determined and optimized for a particular combination of subunits, cells and assay conditions.

In particular, mammalian cells have been transiently and stably transfected with DNA encoding one or more human calcium channel subunits. Such cells express heterologous calcium channels that exhibit pharmacological and electrophysiological properties that can be ascribed to human calcium channels. Such cells, however, represent homogeneous populations and the pharmacological and electrophysiological data provides insights into human calcium channel activity heretofore unattainable. For example, HEK cells that have been transiently transfected with DNA encoding the  $\alpha_{1E-1}$ ,  $\alpha_{2b}$ , and  $\beta_{1-3}$  subunits. The resulting cells transiently express these subunits, which form calcium channels that have properties that appear to be a pharmacologically distinct class of voltage-activated calcium channels distinct from those of L-, N-, T- and P-type channels. The observed  $\alpha_{1E}$  currents were insensitive to drugs and toxins previously used to define other classes of voltage-activated calcium channels.

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Preferred among transfected cells is a recombinant eukaryotic cell with a functional heterologous calcium channel. The recombinant cell can be produced by introduction of and expression of heterologous DNA or RNA transcripts encoding an  $\alpha_1$  subunit of a human calcium channel as a

5 homomer, more preferably also expressing, a heterologous DNA encoding a  $\beta$  subunit of a human calcium channel and/or heterologous DNA encoding an  $\alpha_2$  subunit of a human calcium channel. Especially preferred is the expression in such a recombinant cell of each of the  $\alpha_1$ ,  $\beta$  and  $\alpha_2$  subunits encoded by such heterologous DNA or RNA transcripts, and

10 optionally expression of heterologous DNA or an RNA transcript encoding a  $\gamma$  subunit of a human calcium channel. The functional calcium channels may preferably include at least an  $\alpha_1$  subunit and a  $\beta$  subunit of a human calcium channel. Eukaryotic cells expressing these two subunits and also cells expressing additional subunits, have been prepared by

15 transfection of DNA and by injection of RNA transcripts. Such cells have exhibited voltage-dependent calcium channel activity attributable to calcium channels that contain one or more of the heterologous human calcium channel subunits. For example, eukaryotic cells expressing heterologous calcium channels containing an  $\alpha_2$  subunit in addition to the

20  $\alpha_1$  subunit and a  $\beta$  subunit have been shown to exhibit increased calcium selective ion flow across the cellular membrane in response to depolarization, indicating that the  $\alpha_2$  subunit may potentiate calcium channel function. Cells that have been co-transfected with increasing ratios of  $\alpha_2$  to  $\alpha_1$  and the activity of the resulting calcium channels has

25 been measured. The results indicate that increasing the amount of  $\alpha_2$ -encoding DNA relative to the other transfected subunits increases calcium channel activity.

Eukaryotic cells that express heterologous calcium channels containing a human  $\alpha_1$  subunit as a homomer, particularly the  $\alpha_{1H}$  subunit,

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functional calcium channels and to characterize the kinetics and other characteristics of the resulting currents. Pharmacological studies may be combined with the electrophysiological measurements in order to further characterize the calcium channels.

- 5           With respect to measurement of the activity of functional heterologous calcium channels, preferably, endogenous ion channel activity and, if desired, heterologous channel activity of channels that do not contain the desired subunits, of a host cell can be inhibited to a significant extent by chemical, pharmacological and electrophysiological means, including  
10 the use of differential holding potential, to increase the S/N ratio of the measured heterologous calcium channel activity.

- Thus, various combinations of subunits encoded by the DNA provided herein are introduced into eukaryotic cells. The resulting cells can be examined to ascertain whether functional channels are expressed  
15 and to determine the properties of the channels. In particularly preferred aspects, the eukaryotic cell which contains the heterologous DNA expresses it and forms a recombinant functional calcium channel activity. In more preferred aspects, the recombinant calcium channel activity is readily detectable because it is a type that is absent from the  
20 untransfected host cell or is of a magnitude and/or pharmacological properties or exhibits biophysical properties not exhibited in the untransfected cell.

- The eukaryotic cells can be transfected with various combinations of the subunit subtypes provided herein. The resulting cells will provide a  
25 uniform population of calcium channels for study of calcium channel activity and for use in the drug screening assays provided herein. Experiments that have been performed have demonstrated the inadequacy of prior classification schemes.

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cell are also provided. Substantially pure subtype- or tissue-type specific calcium channels are also provided.

In one embodiment, eukaryotic cells that contain heterologous DNA encoding at least one of  $\alpha_1$  subunit of a calcium channel, preferably an  $\alpha_{1H}$  subunit, that express the  $\alpha_{1H}$  subunit and form functional homomeric human  $\alpha_{1H}$ -containing calcium channels are provided. These cells may be used to screen for compounds that modulate the activity of T-type channels and LVA type calcium channels.

In other embodiments, eukaryotic cells that contain heterologous DNA encoding at least one of an  $\alpha_1$  subunit of a human calcium channel, an  $\alpha_2$  subunit of a human calcium channel, a  $\beta$  subunit of a human calcium channel and a  $\gamma$  subunit of a human calcium channel are provided. In accordance with one preferred embodiment, the heterologous DNA is expressed in the eukaryotic cell and preferably encodes a human calcium channel  $\alpha_1$  subunit.

#### Expression of heterologous calcium channels: electrophysiology and pharmacology

The  $\alpha_{1H-1}$  subunit-encoding DNA was transiently expressed in HEK203 cells and associated with expression of an  $\alpha_{1H-1}$  protein of approximately 260kDa  $\alpha_{1H-1}$ , as identified by SDS-PAGE/Western blot analysis.

$Ba^{2+}$  or  $Ca^{2+}$  currents recorded from HEK293 cells transiently expressing  $\alpha_{1H-1}$  channels, and found to exhibit biophysical and pharmacological properties characteristic of low-voltage activated, i.e., T-type, calcium channel currents. Similar results were obtained in *Xenopus* oocytes expressing  $\alpha_{1H-1}$ .

Electrophysiological methods for measuring calcium channel activity are known to those of skill in the art and are exemplified herein. Any such methods may be used in order to detect the formation of

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- Pichia pastoris*. Preferred cells for expressing injected RNA transcripts or cDNA include *Xenopus laevis* oocytes. Cells that are preferred for transfection of DNA are those that can be readily and efficiently transfected. Such cells are known to those of skill in the art or may be empirically identified. Preferred cells include DG44 cells and HEK 293 cells, particularly HEK 293 cells that can be frozen in liquid nitrogen and then thawed and regrown. Such HEK 293 cells are described, for example in U.S. Patent No. 5,024,939 to Gorman (see, also Stillman *et al.* (1985) *Mol. Cell. Biol.* 5:2051-2060).
- 10 The cells may be used as vehicles for replicating heterologous DNA introduced therein or for expressing the heterologous DNA introduced therein. In certain embodiments, the cells are used as vehicles for expressing the heterologous DNA as a means to produce substantially pure human calcium channel subunits or heterologous calcium channels.
- 15 Host cells containing the heterologous DNA may be cultured under conditions whereby the calcium channels are expressed. The calcium channel subunits may be purified using protein purification methods known to those of skill in the art. For example, antibodies, such as those provided herein, that specifically bind to one or more of the subunits may
- 20 be used for affinity purification of the subunit or calcium channels containing the subunits.

Substantially pure subunits of a human calcium channel  $\alpha_1$  subunits of a human calcium channel,  $\alpha_2$  subunits of a human calcium channel,  $\beta$  subunits of a human calcium channel and  $\gamma$  subunits of a human calcium

25 channel are provided. Substantially pure isolated calcium channels that contain at least one of the human calcium channel subunits are also provided. Substantially pure calcium channels that contain a mixture of one or more subunits encoded by the host cell and one or more subunits encoded by heterologous DNA or RNA that has been introduced into the



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Stably or transiently transfected mammalian cells may be prepared by methods known in the art by transfecting cells with an expression vector having a selectable marker gene such as the gene for thymidine kinase, dihydrofolate reductase, neomycin resistance or the like, and, for  
5 transient transfection, growing the transfected cells under conditions selective for cells expressing the marker gene. Functional voltage-dependent calcium channels have been produced in HEK 293 cells transfected with a derivative of the vector pCDNA1 that contains DNA encoding a human calcium channel subunit.

10 The heterologous DNA may be maintained in the cell as an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case of mammalian cells) from such a culture or a subculture thereof. Methods for transfection, injection and culturing  
15 recombinant cells are known to the skilled artisan. Eukaryotic cells in which DNA or RNA may be introduced, include any cells that are transfectable by such DNA or RNA or into which such DNA may be injected. Virtually any eukaryotic cell can serve as a vehicle for heterologous DNA. Preferred cells are those that can also express the  
20 DNA and RNA and most preferred cells are those that can form recombinant or heterologous calcium channels that include one or more subunits encoded by the heterologous DNA. Such cells may be identified empirically or selected from among those known to be readily transfected or injected. Preferred cells for introducing DNA include those that can  
25 be transiently or stably transfected and include, but are not limited to, cells of mammalian origin, such as COS cells, mouse L cells, CHO cells, human embryonic kidney cells, African green monkey cells and other such cells known to those of skill in the art, amphibian cells, such as *Xenopus laevis* oöcytes, or those of yeast such as *Saccharomyces cerevisiae* or

Cloned full-length nucleic acid encoding any of the subunits of a calcium channel may be introduced into a plasmid vector for expression in a eukaryotic cell. Such nucleic acid may be genomic DNA or cDNA or RNA. Presently preferred cells are those containing heterologous DNA  
5 encoding an  $\alpha_{1H}$  subunit. Host cells may be transfected with one or a combination of the plasmids, each of which encodes at least one calcium channel subunit. Alternatively, host cells may be transfected with linear DNA using methods well known to those of skill in the art.

While the DNA provided herein may be expressed in any eukaryotic  
10 cell, including yeast cells such as *P. pastoris* (see, e.g., Cregg *et al.* (1987) *Bio/Technology* 5:479), mammalian expression systems for expression of the DNA encoding the human calcium channel subunits provided herein are preferred.

The heterologous DNA may be introduced by any method known to  
15 those of skill in the art, such as transfection with a vector encoding the heterologous DNA. Particularly preferred vectors for transfection of mammalian cells are the pSV2dhfr expression vectors, which contain the SV40 early promoter, mouse dhfr gene, SV40 polyadenylation and splice sites and sequences necessary for maintaining the vector in bacteria,  
20 cytomegalovirus (CMV) promoter-based vectors such as pCDNA1, or pcDNA-amp and MMTV promoter-based vectors. The vector pcDNA1 is a eukaryotic expression vector containing a cytomegalovirus (CMV) promoter which is a constitutive promoter recognized by mammalian host cell RNA polymerase II. DNA encoding the human calcium channel  
25 subunits has been inserted in the vector pCDNA1 at a position immediately following the CMV promoter. The vector pCDNA1 is presently preferred and has been used to express the  $\alpha_{1H}$  subunits in mammalian cells.

$\alpha$ 1B-1	2164-2339	$\alpha$ 1B-1#3 GST fusion	polyclonal
$\alpha$ 1B-2	2164-2237	$\alpha$ 1B-2#4 GST fusion	polyclonal
$\alpha$ 1E generic	985-1004 ( $\alpha$ 1E-3)	$\alpha$ 1E#2(a) GST fusion	polyclonal

5 \* GST gene fusion system is available from Pharmacia; see also, Smith et al. (1988) *Gene* 67:31. The system provides pGEX plasmids that are designed for inducible, high-level expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST. Upon expression in a bacterial host, the resulting fusion proteins are purified from bacterial lysates by affinity chromatography.

10 The GST fusion proteins are each specific for the cytoplasmic loop region IIS6-IIS1, which is a region of low subtype homology for all subtypes, including  $\alpha_{1C}$  and  $\alpha_{1D}$ , for which similar fusions and antisera can be prepared.

Using similar methods, antibodies specific for LVA subunits, particularly the  $\alpha_{1H}$  subunits provided herein, using, for example, the extended intracellular loops, can be prepared. Such antibodies will have use in diagnostic assays for disorders in which LVA calcium channels are implicated.

20 Preparation of recombinant eukaryotic cells containing DNA encoding heterologous calcium channel subunits

DNA encoding one or more of the calcium channel subunits or a portion of a calcium channel subunit may be introduced into a host cell for expression or replication of the DNA. Such DNA may be introduced using methods described in the following examples or using other procedures well known to those skilled in the art. Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are also well known in the art (see, e.g., Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor Laboratory Press).

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The antibodies can be administered to a subject employing standard methods, such as, for example, by intraperitoneal, intramuscular, intravenous, or subcutaneous injection, implant or transdermal modes of administration. One of skill in the art can

5 empirically determine dosage forms, treatment regimens, and other parameters, depending on the mode of administration employed.

Subunit-specific monoclonal antibodies and polyclonal antisera have been prepared. The regions from which the antigens were derived were identified by comparing the DNA and amino acid sequences of all known

10  $\alpha$  or  $\beta$  subunit subtypes. Regions of least homology, preferably human-derived sequences were selected. The selected regions or fusion proteins containing the selected regions are used as immunogens. Hydrophobicity analyses of residues in selected protein regions and fusion proteins are also performed; regions of high hydrophobicity are avoided.

15 Also, and more importantly, when preparing fusion proteins in bacterial hosts, rare codons are avoided. In particular, inclusion of 3 or more successive rare codons in a selected host is avoided. Numerous antibodies, polyclonal and monoclonal, specific for  $\alpha$  or  $\beta$  subunit types or subtypes have been prepared; some of these are listed in the following

20 Table. Exemplary antibodies and peptide antigens that have been used to prepare the antibodies are set forth Table 3:

TABLE 3

25	SPECIFICITY	AMINO ACID NUMBER	ANTIGEN NAME	ANTIBODY TYPE
	$\alpha$ 1 generic	112-140	peptide 1A#1	polyclonal
	$\alpha$ 1 generic	1420-1447	peptide 1A#2	polyclonal
	$\alpha$ 1A generic	1048-1208	$\alpha$ 1A#2(b) GST fusion	polyclonal
				monoclonal
	$\alpha$ 1B generic	983-1106	$\alpha$ 1B#2(b) GST fusion	polyclonal
				monoclonal

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depolarization of cell membranes containing calcium channels that include different types of  $\alpha_1$  subunits.

DNA that encodes an  $\alpha_1$  subunit that binds to at least one compound selected from among dihydropyridines, phenylalkylamines,  $\omega$ -CgTx, components of funnel web spider toxin, and pyrazonoylguanidines is provided. For example, the  $\alpha_{1B}$  subunit provided herein appears to specifically interact with  $\omega$ -CgTx in N-type channels, and the  $\alpha_{1D}$  subunit provided herein specifically interacts with DHPs in L-type channels.

#### Antibodies

10 Antibodies, monoclonal or polyclonal, specific for calcium channel subunit subtypes or for calcium channel types can be prepared employing standard techniques, known to those of skill in the art, using the subunit proteins or portions thereof as antigens. Anti-peptide and anti-fusion protein antibodies can be used (see, for example, Bahouth et al. (1991) *Trends Pharmacol. Sci.* 12:338-343; *Current Protocols in Molecular Biology* (Ausubel et al., eds.) John Wiley and Sons, New York (1984))

15 Factors to consider in selecting portions of the calcium channel subunits for use as immunogens (as either a synthetic peptide or a recombinantly produced bacterial fusion protein) include antigenicity accessibility (i.e.,

20 extracellular and cytoplasmic domains), uniqueness to the particular subunit, and other factors known to those of skill in this art. Antibodies have therapeutic uses and also use in diagnostic assays.

The availability of subunit-specific antibodies makes possible the application of the technique of immunohistochemistry to monitor the

25 distribution and expression density of various subunits (e.g., in normal vs diseased brain tissue). Such antibodies could also be employed in diagnostic, such as LES diagnosis, and therapeutic applications, such as using antibodies that modulate activities of calcium channels.

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hippocampus, substantia nigra, subthalamic nucleus and thalamus. In nonneuronal tissues, the highest expression levels are found in the kidney, liver, and heart. In the brain, the  $\alpha_{1H}$  transcript is most abundant in the amygdala, caudate nucleus, and putamen.

**5 Identification and isolation of DNA encoding other  $\alpha_1$  human calcium channel subunit types and subtypes**

DNA encoding additional  $\alpha_1$  subunits can be isolated and identified using the DNA provided herein as described for the  $\alpha_{1A}$ ,  $\alpha_{1B}$ ,  $\alpha_{1C}$ ,  $\alpha_{1D}$ ,  $\alpha_{1E}$  and  $\alpha_{1H}$  subunits or using other methods known to those of skill in the art.

**10** In particular, the DNA provided herein may be used to screen appropriate libraries to isolate related DNA. Full-length clones can be constructed using methods, such as those described herein, and the resulting subunits characterized by comparison of their sequences and electrophysiological and pharmacological properties with the subunits exemplified herein.

**15** A number of voltage-dependent calcium channel  $\alpha_1$  subunit genes, which are expressed in the human CNS and in other tissues, have been identified and have been designated as  $\alpha_{1A}$ ,  $\alpha_{1B}$  (or VDCC IV),  $\alpha_{1C}$  (or VDCC II),  $\alpha_{1D}$  (or VDCC III),  $\alpha_{1E}$  and  $\alpha_{1H}$ . DNA, isolated from a human DNA libraries that encodes each of the subunit types has been isolated.

**20** DNA encoding subtypes of each of the types, which arise as splice variants are also provided. Subtypes are herein designated, for example, as  $\alpha_{1B-1}$ ,  $\alpha_{1B-2}$ . The  $\alpha_{1H}$  subunit is of particular interest herein

The  $\alpha_1$  subunit types A, B, C, D, E and F of voltage-dependent calcium channels, and subtypes thereof, differ with respect to sensitivity  
**25** to known classes of calcium channel agonists and antagonists, such as DHPs, phenylalkylamines, omega conotoxins ( $\omega$ -CgTx), the funnel web spider toxin  $\omega$ -Aga-IV, pyrazonoylguanidines and or in other physical and structural properties. These subunit types also appear to differ in the holding potential and in the kinetics of currents produced upon

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The nucleic acid encoding an  $\alpha_{1H}$  subunit can be used to screen appropriate libraries, particularly mammalian libraries, and more particularly mammalian libraries from tissues or cells that exhibit T-type channel activity. The encoded subunit can be identified by the above-

5 noted distinguishing properties. Nucleic acid probes from the  $\alpha_{1H-1}$ -encoding clone was used to identify and isolate clones encoding a second variant, designated  $\alpha_{1H-2}$ , which has a 957 bp deletion relative to  $\alpha_{1H-1}$ .

The  $\alpha_{1H}$  subunit forms a functional channel in two different expression systems without the addition of exogenous  $\alpha_2\delta$  and  $\beta$

10 subunits. The absence of a  $\beta$  subunit interaction site within the I-II loop of the  $\alpha_{1H}$  sequence is consistent with the report that  $\beta$  subunit depletion with antisense oligonucleotides in nodosus ganglia has no effect on T-type currents in that region. In addition, none of the known  $\beta$  subunits in HEK293 cells were detected by western analysis using  $\beta$  subunit-specific

15 antisera, indicating that the previously cloned  $\beta$  subunits may not play a role in the formation of LVA  $Ca^{2+}$  channels containing  $\alpha_{1H}$ . Oocytes and HEK293 cells express an endogenous  $\alpha_2\delta$  subunit and that TT cells, the source of the  $\alpha_{1H}$  subunits described here, express relatively high amounts of  $\alpha_2\delta$  protein. Consequently, it is possible that  $\alpha_{1H}$ -containing channels

20 expressed, contain  $\alpha_2\delta$  subunit, and that the  $\alpha_2\delta$  subunit is a component of native  $\alpha_{1H}$ -containing channels.

#### Distribution of $\alpha_{1H}$ transcripts

Northern blots containing human mRNA from several neuronal and nonneuronal tissues were probed with labeled fragments generated from

25 the full-length  $\alpha_{1H}$  cDNA. A single transcript of  $\sim 8.5$  kb is present in all tissues examined, which included heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas. Neuronal tissues included, cerebellum, cerebral cortex, medulla, spinal cord, occipital lobe, frontal lobe, temporal lobe, putamen, amygdala, caudate nucleus, corpus callosum,

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between HVA calcium channels and sodium channel. The  $\alpha_{1H}$  subunits provided herein may also function as sodium channels.

- Second, the isolated human  $\alpha_{1H}$  subunit lacks amino acid residues that are generally known to be critical (e.g., see De Waard *et al.* (1996) FEBS Letters **380**:272-276; Pragnell *et al.* (1994) Nature **368**:67-70) for the interaction between  $\alpha_1$  subunits and the  $\beta$  subunits. There are at least thirteen residues located in this intracellular loop between transmembrane Domains I and II that form a motif that is highly conserved among  $\alpha_1$  subunits, such as  $\alpha_{1A}$ - $\alpha_{1E}$  described herein (see, also Pragnell *et al.* (1994) Nature **368**:67-70). In particular, this loop lacks the  $\alpha_1$  interaction domain (AID) involved in binding the  $\beta$  subunit. Also absent from this region is the  $G\beta\gamma$  binding motif, GlnXXGluArg, originally identified in adenylyl cyclase 2 and found in the non-L-type, HVA  $\alpha_1$  subunits. An identical sequence occurs, however, within the II-III intracellular loop of the  $\alpha_{1H}$  sequence, suggesting a possible interaction of  $G\beta\gamma$  in this region. The  $\alpha_{1H}$  subunit also contains differences in the determinants of ion selectivity found in the S5-S6 linkers of HVA channels. In the S5-S6 pore loops of domain III and IV, the glutamate residues that play a critical role in  $Ca^{2+}$  selectivity and ion permeation are replaced by aspartate residues.
- Third, the human  $\alpha_{1H}$  subunit has another notably long extracellular loop in Domain I located between IS5 and IS6. This extracellular loop ranges from 249 to 270 nucleotide residues in other human  $\alpha_1$  subunits whereas the human  $\alpha_{1H}$  subunit has 426 nucleotide residues. Other distinguishing features may be ascertained and have been ascertained by expressing the subunit in cells as described herein.



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$\alpha_{1H-2}$  isoform (SEQ ID No. 16) contains a 957 nucleotide deletion, relative to  $\alpha_{1H-1}$  (SEQ ID Nos. 12 and 15) in the I-II intracellular loop, i.e., (e.g., nt 1506 to nt 2627 of SEQ ID No. 12).

The  $\alpha_{1H-1}$  subunit exhibits marked sequence differences, as well as  
5 certain structural similarities to previously cloned  $\alpha_1$  subunits. Notably, the deduced amino acid sequence of  $\alpha_{1H-1}$  shares less than 30% overall sequence identity with human  $\alpha_{1A}$ - $\alpha_{1E}$ -encoding nucleic acids, which encode high-voltage activated calcium channels. Northern blot analysis indicates that mRNA transcripts for  $\alpha_{1H}$  are expressed in the brain,  
10 primarily in the amygdala, caudate nucleus and putamen, and in peripheral tissues, primarily in the liver, kidney and heart.

Specifically, a comparison of the nucleic acid and deduced amino acid sequences of this  $\alpha_{1H}$  calcium channel subunit with other human  $\alpha_1$  subunits reveals several distinct features. There are notable differences  
15 between  $\alpha_{1H}$  and the HVA  $\alpha_1$  sequences. First, the intracellular loop between transmembrane Domains I and II is notably long. As exemplified in SEQ ID No. 49, the intracellular loop of human  $\alpha_{1H}$  subunit is 1,122 nt in length whereas the corresponding intracellular loops in the other human  $\alpha_1$  subunits described herein range from 351 to 381 nt in length. Thus,  
20 the intracellular loop of human  $\alpha_{1H}$  is nearly 250 amino acids longer than human  $\alpha_1$  subunits found in HVA calcium channels. The deduced amino acid sequence of this region (aa 420 to aa 794 of SEQ ID No. 12) contains a large number of proline residues and includes a poly-HIS region of 9 contiguous histidine residues (aa 52 to aa 528 of SEQ ID No. 12)  
25 and a region where 8 of 10 residues are alanine. The large intracellular loop located between transmembrane Domains I and II resembles the large intracellular loops found in a corresponding location in sodium channel  $\alpha$  subunits some of which may function as homomers. It has been proposed that T-type channels have an activity that is a hybrid

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DNA encoding an  $\alpha_{1H}$ -subunit may be isolated using the DNA provided herein. In particular, probes of at least about 16 nucleotides or 30 nucleotides or other suitable length, such 14, 30, 100 etc. bases, may be used to screen selected libraries, including mammalian DNA libraries.

5 The selected libraries are preferably prepared from mammalian tissue or cell sources known to express T-type channels. The sequence of the probe is preferably based on the sequence of the intracellular loop located between transmembrane domains I and II (see, e.g., SEQ ID Nos. 12 and 15).

10 DNA encoding the  $\alpha_{1H}$  subunit was isolated by amplifying a region of genes encoding an  $\alpha_1$  subunit expressed in a human thyroid carcinoma cell line (TT cells) using degenerate oligonucleotide primers.

The TT cell line is derived from a human medullary thyroid carcinoma and has been used to study calcitonin secretion and gene expression

15 (deBustros et al. (1986) J. Biol. Chem. 261:8036-8041; deBustros et al. 1990 Mol. Cell. Biol. 10:1773-1778). Whole-cell recordings from these cells reveal that the only voltage gated calcium channels expressed by these cells are low-voltage activated, rapidly inactivating and slowly deactivating, which are biophysical properties consistent with a T-type  
20 channel.

A portion of one of the positive clones was used to further screen a human thyroid carcinoma cDNA library to identify overlapping clones that span the entire length of the nucleotide sequence encoding the human  $\alpha_{1H}$  subunit. A full-length  $\alpha_{1H}$  DNA clone can be constructed by ligating  
25 portions of the partial cDNA clones as described in Example 1. SEQ ID No. 15 sets forth the nucleotide sequence of a clone encoding an  $\alpha_{1H.1}$  subunit as well as the deduced amino acid sequence.

Two splice variants,  $\alpha_{1H.1}$  and  $\alpha_{1H.2}$ , were detected by RT-PCR (reverse transcriptase-amplification) using RNA from multiple tissues. The

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understood that the exemplified methods may be used to isolate additional splice variants and related subunits from humans and other mammals and animals and may also be used to express such nucleic acid to produce cells for use in screening assays to identify compounds that

5 modulate the activity of LVA activated channels, particularly T-type channels. The nucleic acid may also be used in diagnostic assays to identify mutations and to produce proteins and then antibodies for use as reagents in diagnostic assays for disorders associated with T-type calcium channel activities.

10  **$\alpha_1$  subunits of LVA channels**

Nucleic acid encoding  $\alpha_1$  subunits that form LVA channels is provided herein. The nucleic acid provided herein may also be used to isolate related channels from other tissues, and other mammals and animals.

15 **Identification and isolation of DNA encoding the  $\alpha_{1H}$  human calcium channel subunits**

Calcium channels that contain  $\alpha_{1H}$  should exhibit properties that differ from known HVA channels, formed from the  $\alpha_{1A}$  -  $\alpha_{1E}$  calcium channel subunits. Such differences may include low voltage activation,

20 voltage-dependent inactivation, relatively high sensitivity to mibefradil and relatively high resistance to snail and arachnid toxins that inhibit most HVA channels (e.g., spider venom toxins  $\omega$ -AgallIA and  $\omega$ -AgalVA and the Conus snail toxin GVIA). In addition  $\alpha_{1H}$ -subunits may be identified by homology with other  $\alpha_1$ -subunits and additionally by presence of an

25 extended intracellular loop in the encoded subunit (see, e.g., SEQ No. 49, nucleotides 1506-2627) located between transmembrane domains I and II. This region in  $\alpha_{1H}$  is extended compared to other calcium channel  $\alpha_1$  subunits, such as  $\alpha_{1A}$ - $\alpha_{1E}$ .

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single-stranded RNA. Any RNA-DNA hybrids are protected from RNase degradation and can be visualized by gel electrophoresis and autoradiography. *In situ* hybridization techniques can also be used to determine which tissues express mRNA encoding a particular calcium channel subunit. The labeled subunit-encoding DNA clones are hybridized to different tissue slices to visualize subunit mRNA expression.

With respect to each of the respective subunits ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta$  or  $\gamma$ ) of human calcium channels, once the DNA encoding the channel subunit was identified by a nucleic acid screening method, the isolated clone was used for further screening to identify overlapping clones. Some of the cloned DNA fragments can and have been subcloned into an appropriate vector such as pIBI24/25 (IBI, New Haven, CT), M13mp18/19, pGEM4, pGEM3, pGEM7Z, pSP72 and other such vectors known to those of skill in this art, and characterized by DNA sequencing and restriction enzyme mapping. A sequential series of overlapping clones may thus be generated for each of the subunits until a full-length clone can be prepared by methods, known to those of skill in the art, that include identification of translation initiation (start) and translation termination (stop) codons. For expression of the cloned DNA, the 5' noncoding region and other transcriptional and translational control regions of such a clone may be replaced with an efficient ribosome binding site and other regulatory regions as known in the art. Other modifications of the 5' end, known to those of skill in the art, that may be required to optimize translation and/or transcription efficiency may also be effected, if deemed necessary.

Examples 1-3 below, describe in detail the cloning DNA encoding  $\alpha_{1H}$  splice variants and electrophysiological and pharmacological properties thereof. Except where noted, the methods of expression and other data is described with reference to the  $\alpha_{1H-1}$  encoding nucleic acid. It is

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$\alpha$ 1B-1 specific	6490-6676	pCRII $\alpha$ 1B-1/187	riboprobe	n/a
$\alpha$ 1E generic	3114-3462	pGEM7Z $\alpha$ 1E	riboprobe	n/a

5 \* The pGEM series are available from Promega, Madison WI; see also, U.S. Patent No. 4,766,072.

For the  $\alpha_{1H}$ -specific probes (and also antibodies), regions unique to the  $\alpha_{1H}$  subunits, such as the extended intracellular loops present in these channels may be used. For  $\alpha_{1H-1}$  specific antibodies the region present in  $\alpha_{1H-1}$  and absent from  $\alpha_{1H-2}$  may be useful for preparation of subunit-

10 specific probes. purpose.

The DNA clones and fragments thereof provided herein thus can be used to isolate genomic clones encoding each subunit and to isolate any splice variants by hybridization screening of libraries prepared from different human tissues. Nucleic acid amplification techniques, which are well known in the art, can also be used to locate DNA encoding splice variants of human calcium channel subunits. This is accomplished by employing oligonucleotides based on DNA sequences surrounding divergent sequence(s) as primers for amplifying human RNA or genomic DNA. Size and sequence determinations of the amplification products can reveal the existence of splice variants. Furthermore, isolation of human genomic DNA sequences by hybridization can yield DNA containing multiple exons, separated by introns, that correspond to different splice variants of transcripts encoding human calcium channel subunits.

Once DNA encoding a calcium channel subunit is isolated, ribonuclease (RNase) protection assays can be employed to determine which tissues express mRNA encoding a particular calcium channel subunit or variant. These assays provide a sensitive means for detecting and quantitating an RNA species in a complex mixture of total cellular RNA. The subunit DNA is labeled and hybridized with cellular RNA. If complementary mRNA is present in the cellular RNA, a DNA-RNA hybrid results. The RNA sample is then treated with RNase, which degrades

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domains, sequences predicted to encode cytoplasmic loops, signal sequences, ligand-binding sites, and other functionally significant sequences (see Table, below). Either the full-length subunit-encoding DNA or fragments thereof can be used as probes, preferably labeled with  
 5 suitable label means for ready detection. When fragments are used as probes, preferably the DNA sequences will be typically from the carboxyl-end-encoding portion of the DNA, and most preferably will include predicted transmembrane domain-encoding portions based on hydropathy analysis of the deduced amino acid sequence (see, e.g., Kyte and  
 10 Doolittle ((1982) *J. Mol. Biol.* 167:105).

Riboprobes that are specific for human calcium channel subunit types or subtypes have been prepared. These probes are useful for identifying expression of particular subunits in selected tissues and cells. The regions from which the probes were prepared were identified by  
 15 comparing the DNA and amino acid sequences of all known  $\alpha$  or  $\beta$  subunit subtypes. Regions of least homology, preferably human-derived sequences, and generally about 250 to about 600 nucleotides were selected. Numerous riboprobes for  $\alpha$  and  $\beta$  subunits have been prepared (see, e.g., Table 2 in International PCT application No. WO95/04822),  
 20 which is repeated in part in the following Table.

**TABLE 2**  
**SUMMARY OF RNA PROBES**

	SUBUNIT SPECIFICITY	NUCLEOTIDE POSITION	PROBE NAME	PROBE TYPE	ORIENTATION
25	$\alpha$ 1A generic	3357-3840	pGEM7Z $\alpha$ 1A'	riboprobe	n/a
		761-790	SE700	oligo	antisense
		3440-3464	SE718	oligo	antisense
		3542-3565	SE724	oligo	sense
30	$\alpha$ 1B generic	3091-3463	pGEM7Z $\alpha$ 1B <sub>cyt</sub>	riboprobe	n/a
		6635-6858	pGEM7Z $\alpha$ 1B <sub>cooh</sub>	riboprobe	n/a

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to a segment of a nucleic acid sequence disclosed herein by reference to a SEQ ID No. are provided. Such nucleic acid segments may be used as probes in the methods provided herein for cloning DNA encoding calcium channel subunits. See, generally, Sambrook *et al.* (1989) *Molecular*

5 *Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press.

In addition, nucleic acid amplification techniques, which are well known in the art, can be used to locate splice variants of calcium channel subunits by employing oligonucleotides based on DNA sequences

10 surrounding the divergent sequence primers for amplifying human RNA or genomic DNA. Size and sequence determinations of the amplification products can reveal splice variants. Furthermore, isolation of human genomic DNA sequences by hybridization can yield DNA containing multiple exons, separated by introns, that correspond to different splice  
15 variants of transcripts encoding human calcium channel subunits.

DNA encoding types and subtypes of each of the  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$  and  $\gamma$  subunits of voltage-dependent human calcium channels has been cloned by nucleic acid amplification of cDNA from selected tissues or by screening human cDNA libraries prepared from isolated poly A + mRNA  
20 from cell lines or tissue of human origin having such calcium channels. Among the sources of such cells or tissue for obtaining mRNA are human brain tissue or a human cell line of neural origin, such as a neuroblastoma cell line, human skeletal muscle or smooth muscle cells, and the like. Methods of preparing cDNA libraries are well known in the art (see  
25 generally Ausubel *et al.* (1987) *Current Protocols in Molecular Biology*, Wiley-Interscience, New York; and Davis *et al.* (1986) *Basic Methods in Molecular Biology*, Elsevier Science Publishing Co., New York).

Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode transmembrane

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splicing of the primary transcript of genomic subunit DNA. For instance, DNA, cDNA or genomic DNA, encoding a calcium channel subunit may be identified by hybridization to a DNA probe and characterized by methods known to those of skill in the art, such as restriction mapping and DNA sequencing, and compared to the DNA provided herein in order to identify heterogeneity or divergence in the sequences of the DNA. Such sequence differences may indicate that the transcripts from which the cDNA was produced result from alternative splicing of a primary transcript, if the non-homologous and homologous regions are clustered, or from a different gene if the non-homologous regions are distributed throughout the cloned DNA. Splice variants share regions of 100% homology. As noted herein, the resulting nucleic acid may be expressed in cells and the resulting cells tested to verify or ascertain that expressed calcium channels exhibit pharmacological and/or electrophysiological properties of LVA or T-channels.

Any suitable method for isolating genes using the DNA provided herein may be used. For example, oligonucleotides corresponding to regions of sequence differences have been used to isolate, by hybridization, DNA encoding the full-length splice variant and can be used to isolate genomic clones. A probe, based on a nucleotide sequence disclosed herein, which encodes at least a portion of a subunit of a human calcium channel, such as a tissue-specific exon, may be used as a probe to clone related DNA, to clone a full-length cDNA clone or genomic clone encoding the human calcium channel subunit.

Labeled, including, but not limited to, radioactively or enzymatically labeled, RNA or single-stranded DNA of at least 14 substantially contiguous bases, preferably 16 or more, generally at least 30 contiguous bases of a nucleic acid which encodes at least a portion of a human calcium channel subunit, the sequence of which nucleic acid corresponds



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substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers. In such instances, further purification might increase the specific activity of the compound.

5           As used herein, biological activity refers to the in vivo activities of a compound or physiological responses that result upon in vivo administration of a compound, composition or other mixture. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures.

10   **Identification and isolation of DNA encoding human calcium channel subunits**

          Methods for identifying and isolating nucleic acid (DNA and RNA) encoding  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$  and  $\gamma$ , particularly nucleic acid encoding LVA  $\alpha_1$  subunits of human calcium channels are provided.

15           Identification and isolation of such nucleic acid may be accomplished by hybridizing, under appropriate conditions, at least low stringency, preferably high stringency, to restriction enzyme-digested human DNA with a labeled probe having at least 14, preferably 16 or more nucleotides (25, 30 or longer) and derived from any contiguous  
20   portion of DNA having a sequence of nucleotides set forth herein by sequence identification number. Once a hybridizing fragment is identified in the hybridization reaction, it can be cloned employing standard cloning techniques known to those of skill in the art. Full-length clones may be identified by the presence of a complete open reading frame and the  
25   identity of the encoded protein verified by sequence comparison with the subunits provided herein and by functional assays to assess calcium channel- forming ability or other function. This method can be used to identify genomic DNA encoding the subunit or cDNA encoding splice variants of human calcium channel subunits generated by alternative

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directed mutagenesis of DNA encoding the protein and the use of DNA amplification methods using primers to introduce and amplify alterations in the DNA template.

As used herein, treatment means any manner in which the  
5 symptoms of a conditions, disorder or disease are ameliorated or otherwise beneficially altered. Treatment also encompasses any pharmaceutical use of the compositions herein, such as use as contraceptive agents.

As used herein, a LVA-activated calcium channel-mediated disorder  
10 refers to disorders that are associated with LVA channel activities. A T-type calcium channel-mediated disorders LVA-activated channel-mediated disorders that are associated with T-type channels. Such disorders include, but are not limited to: cardiovascular, hepatic, endocrine, urologic, reproductive, muscular, neurological and other disorders in  
15 which LVA channels, particular T-type channels, play a role either in mediating the disorder in some manner contributing to it.

As used herein, amelioration of the symptoms of a particular disorder by administration of a particular pharmaceutical composition refers to any lessening, whether permanent or temporary, lasting or  
20 transient that can be attributed to or associated with administration of the composition.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel  
25 electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce

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It is also understood that each of the subunits disclosed herein may be modified by making conservative amino acid substitutions and the resulting modified subunits are contemplated herein. Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. *Molecular Biology of the Gene*, 4th Edition, 1987, The Benjamin/Cummings Pub. Co., p.224). Such substitutions are preferably, although not exclusively, made in accordance with those set forth in TABLE 1 as follows:

TABLE 1		
	Original residue	Conservative substitution
15	Ala (A)	Gly; Ser
	Arg (R)	Lys
	Asn (N)	Gln; His
	Cys (C)	Ser
20	Gln (Q)	Asn
	Glu (E)	Asp
	Gly (G)	Ala; Pro
	His (H)	Asn; Gln
	Ile (I)	Leu; Val
25	Leu (L)	Ile; Val
	Lys (K)	Arg; Gln; Glu
	Met (M)	Leu; Tyr; Ile
	Phe (F)	Met; Leu; Tyr
	Ser (S)	Thr
30	Thr (T)	Ser
	Trp (W)	Tyr
	Tyr (Y)	Trp; Phe
	Val (V)	Ile; Leu

Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions. Any such modification of the polypeptide may be effected by any means known to those of skill in this art. Mutation may be effected by any method known to those of skill in the art, including site-specific or site-

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ion is a concentration of the calcium channel-selective ion necessary and sufficient to provide an inward current when the channels open.

As used herein, activity of a calcium channel refers to the movement of a calcium channel-selective ion through a calcium channel.

- 5 Such activity may be measured by any method known to those of skill in the art, including, but not limited to, measurement of the amount of current which flows through the recombinant channel in response to a stimulus.

- As used herein, a "functional assay" refers to an assay that  
10 identifies functional calcium channels. A functional assay, thus, is an assay to assess function.

- As understood by those skilled in the art, assay methods for identifying compounds, such as antagonists and agonists, that modulate calcium channel activity, generally require comparison to a control. One  
15 type of a "control" cell or "control" culture is a cell or culture that is treated substantially the same as the cell or culture exposed to the test compound except that the control culture is not exposed to the test compound. Another type of a "control" cell or "control" culture may be a cell or a culture of cells which are identical to the transfected cells except  
20 the cells employed for the control culture do not express functional calcium channels. In this situation, the response of test cell to the test compound is compared to the response (or lack of response) of the calcium channel-negative cell to the test compound, when cells or cultures of each type of cell are exposed to substantially the same  
25 reaction conditions in the presence of the compound being assayed. For example, in methods that use patch clamp electrophysiological procedures, the same cell can be tested in the presence and absence of the test compound, by changing the external solution bathing the cell as known in the art.

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As used herein, a T-type channel or LVA type channel typically refers to a calcium channel that exhibits a low-threshold calcium current that is activated and inactivated at low voltages compared to calcium channels (such as those that include an  $\alpha_{1D}$  subunit) referred to as high voltage activated (HVA) channels. In addition or alternatively, a T-type channel may be characterized by distinct biophysical features, such as slow deactivation rates, very low conductances (5-9 pS) and voltage-dependent inactivation. T channels may exhibit a relatively high degree of sensitivity to mibefradil (Hoffman-LaRoche, Inc.) and/or a relatively high degree of resistance to the Conus snail toxins GVIA and MVIIC as well as the arachnid toxins AgallIA and AgalVA compared to HVA calcium channels. These channels also typically exhibit reduced affinity for cadmium. T-type channels or LVA type channels may also be characterized at the nucleic acid level by the presence of one or more extended intracellular loops (see, e.g., SEQ ID NO. 12, 15 and 16) between transmembrane domains, such as between transmembrane domains I and II.

As used herein, a polypeptide having an amino acid sequence substantially as set forth in a particular SEQ ID No. includes protein that may have the same function but may include minor variations in sequence, such as conservative amino acid changes or minor deletions or insertions that do not alter the activity of the protein. The activity of a calcium channel receptor subunit protein, particularly a LVA or T-type channel, refers to its ability to form a functional calcium channel alone or with other subunits. A T-type channel will have the distinguishing properties defined herein.

As used herein, a physiological concentration of a compound is that which is necessary and sufficient for a biological process to occur. For example, a physiological concentration of a calcium channel I-selective

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As used herein, a promoter region refers to the portion of DNA of a gene that controls transcription of the DNA to which it is operatively linked. The promoter region includes specific sequences of DNA that are sufficient for RNA polymerase recognition, binding and transcription  
5 initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of the RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature  
10 of the regulation, may be constitutive or regulated.

As used herein, a recombinant eukaryotic cell is a eukaryotic cell that contains heterologous DNA or RNA.

As used herein, a recombinant or heterologous calcium channel refers to a calcium channel that contains one or more subunits that are  
15 encoded by heterologous DNA that has been introduced into and expressed in a eukaryotic cell that expresses the recombinant calcium channel. A recombinant calcium channel may also include subunits that are produced by DNA endogenous to the cell. In certain embodiments, the recombinant or heterologous calcium channel may contain only  
20 subunits that are encoded by heterologous DNA.

As used herein, "functional" with respect to a recombinant or heterologous calcium channel means that the channel is able to provide for and regulate entry of calcium channel-selective ions, including, but not limited to,  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$ , in response to a stimulus and/or bind ligands  
25 with affinity for the channel. Preferably such calcium channel activity is distinguishable, such as by electrophysiological, pharmacological and other means known to those of skill in the art, from any endogenous calcium channel activity that is in the host cell.

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transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA in reading frame.

As used herein, isolated, substantially pure DNA refers to DNA  
5 fragments purified according to standard techniques employed by those skilled in the art (see, *e.g.*, Maniatis *et al.* (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

As used herein, expression refers to the process by which nucleic  
10 acid is transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the nucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

As used herein, vector or plasmid refers to discrete elements that  
15 are used to introduce heterologous DNA into cells for either expression of the heterologous DNA or for replication of the cloned heterologous DNA. Selection and use of such vectors and plasmids are well within the level of skill of the art.

As used herein, expression vector includes vectors capable of  
20 expressing DNA fragments that are in operative linkage with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an  
25 appropriate host cell, results in expression of the cloned DNA.

Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or may integrate into the host cell genome.

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contaminants to appear homogeneous by SDS-PAGE or to be unambiguously sequenced.

As used herein, selectively hybridize means that a DNA fragment hybridizes to a second fragment with sufficient specificity to permit the  
5 second fragment to be identified or isolated from among a plurality of fragments. In general, selective hybridization occurs at conditions of high stringency.

As used herein, heterologous or foreign DNA and RNA are used interchangeably and refer to DNA or RNA that does not occur naturally as  
10 part of the genome in which it is present or which is found in a location or locations in the genome that differ from that in which it occurs in nature. It is DNA or RNA that is not endogenous to the cell and has been artificially introduced into the cell. Examples of heterologous DNA  
15 subunit and DNA that encodes RNA or proteins that mediate or alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes. The cell that expresses the heterologous DNA, such as DNA encoding a calcium channel subunit,  
20 subunits. The heterologous DNA need not be expressed and may be introduced in a manner such that it is integrated into the host cell genome or is maintained episomally.

As used herein, operative linkage of heterologous DNA to regulatory and effector sequences of nucleotides, such as promoters,  
25 enhancers, transcriptional and translational stop sites, and other signal sequences, refers to the functional relationship between such DNA and such sequences of nucleotides. For example, operative linkage of heterologous DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the



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library with DNA probes prepared from nucleic acid provided herein. Full-length clones are constructed and expressed as described and exemplified herein and the resulting channels tested to verify that the encoding nucleic acid encodes a LVA channel.

5           The subunit encoded by isolated DNA may be identified by comparison with the DNA and amino acid sequences of the subunits provided herein. Splice variants share extensive regions of homology, but include non-homologous regions, subunits encoded by different genes share a uniform distribution of non-homologous sequences.

10           As used herein, a splice variant refers to a variant produced by differential processing of a primary transcript of genomic DNA that results in more than one type of mRNA. Splice variants may occur within a single tissue type or among tissues (tissue-specific variants). Thus, cDNA clones that encode calcium channel subunit subtypes that have  
15 regions of identical amino acids and regions of different amino acid sequences are referred to herein as "splice variants".

          As used herein, a "calcium channel-selective ion" is an ion that is capable of flowing through, or being blocked from flowing through, a calcium channel which spans a cellular membrane under conditions which  
20 would substantially similarly permit or block the flow of  $\text{Ca}^{2+}$ .  $\text{Ba}^{2+}$  is an example of an ion which is a calcium channel-selective ion.

          As used herein, a compound that modulates calcium channel activity is one that affects the ability of the calcium channel to pass calcium channel-selective ions or affects other detectable calcium channel  
25 features, such as current kinetics. Such compounds include calcium channel antagonists and agonists and compounds that exert their effect on the activity of the calcium channel directly or indirectly.

          As used herein, a "substantially pure" subunit or protein is a subunit or protein that is sufficiently free of other polypeptide

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Thus, one of skill in the art, in light of the disclosure herein, can identify DNA encoding  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ ,  $\delta$  and  $\gamma$  calcium channel subunits, including types encoded by different genes and subtypes that represent splice variants. For example, DNA or RNA probes based on the DNA disclosed herein may be used to screen an appropriate library, including a genomic or cDNA library, for hybridization to the probe and obtain DNA in one or more clones that includes an open reading fragment that encodes an entire protein. Subsequent to screening an appropriate library with the DNA disclosed herein, the isolated DNA can be examined for the presence of an open reading frame from which the sequence of the encoded protein may be deduced. Determination of the molecular weight and comparison with the sequences herein should reveal the identity of the subunit as an  $\alpha_1$ ,  $\alpha_2$  etc. subunit. Functional assays may, if necessary, be used to determine whether the subunit is an  $\alpha_1$ ,  $\alpha_2$  subunit or  $\beta$  subunit.

For example, DNA encoding an  $\alpha_{1A}$  subunit may be isolated by screening an appropriate library with DNA, encoding all or a portion of the human  $\alpha_{1A}$  subunit. Such DNA includes the DNA in the phage deposited under ATCC Accession No. 75293 that encodes a portion of an  $\alpha_1$  subunit. DNA encoding an  $\alpha_{1A}$  subunit may be obtained from an appropriate library by screening with an oligonucleotide having all or a portion of the sequence of an  $\alpha_{1A}$  subunit (see, *e.g.*, published International PCT application No. WO95/04822, particularly SEQ ID Nos. 21, 22 and/or 23 or with the DNA in the deposited phage therein). Alternatively, such DNA may have the coding sequence that encodes an  $\alpha_{1A}$  subunit. Any method known to those of skill in the art for isolation and identification of DNA and preparation of full-length genomic or cDNA clones, including methods exemplified herein, may be used.

DNA encoding  $\alpha_{1H}$  can be isolated by screening a human medullary thyroid carcinoma cell line (TT cells) or other suitable library human cDNA

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stringency, or encodes a protein that has at least about 40% homology, typically about at least about 90% homology) with that disclosed therein and is a protein that typically has a molecular mass lower than the  $\alpha$  subunits and on the order of about 50-80 kD, does not form a detectable  
5 calcium channel in the absence of an  $\alpha_1$  subunit, but may alter the activity of a calcium channel that contains an  $\alpha_1$  subunit or that contains an  $\alpha_1$  and  $\alpha_2$  subunit.

Types of the  $\beta$  subunit that are encoded by different genes are designated with subscripts, such as  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$  and  $\beta_4$ . Subtypes of  $\beta$   
10 subunits that arise as splice variants of a particular type are designated with a numerical subscript referring to the type and to the variant. Such subtypes include, but are not limited to the  $\beta_1$  splice variants, including  $\beta_{1-1}$ ,  $\beta_{1-5}$  and  $\beta_2$  variants, including  $\beta_{2C}$ - $\beta_{2E}$ .

As used herein, a  $\gamma$  subunit is a subunit of calcium channel  
15 encoded by DNA disclosed for example in U.S. Patent Nos. 5,726,035 and 5,386,025; see, also Jay *et al.* (1990) Science 248:490-492 and Lett *et al.* (\*1998) Nature Genetics 19:340-347) and may be isolated and identified using the nucleic disclosed therein as a probe by hybridization or other such method known to those of skill in the art, whereby full-  
20 length clones encoding a  $\gamma$  subunit may be isolated or constructed. A  $\gamma$  subunit will be encoded by nucleic acid that hybridizes to the DNA provided therein under conditions of low stringency, preferably high stringency, exhibits sufficient sequence homology to encode a protein that has at least about 40% homology with the  $\gamma$  subunit described  
25 herein.

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snail toxins GVIA and MVIIC as well as the arachnid toxins AgallIA and AgalVA compared to HVA calcium channels.

As used herein, an  $\alpha_2$  subunit is encoded by nucleic acid (DNA or RNA) disclosed, for example, in U.S. Patent No. 5,407,820, U.S. Patent  
5 No. 5,792,846 and International PCT application No. WO95/04822 that encodes an  $\alpha_2$  subunit of a mammalian calcium channel or that hybridizes to DNA under conditions of low stringency, preferably high stringency, or encodes a protein that has at least about 40% homology, typically at least about 90% identity, taking into account gaps, with that disclosed  
10 therein. Such DNA encodes a protein that typically has a molecular mass greater than about 120 kD, but does not form a calcium channel in the absence of an  $\alpha_1$  subunit, and may alter the activity of a calcium channel that contains an  $\alpha_1$  subunit. Subtypes of the  $\alpha_2$  subunit that arise as splice variants are designated by lower case letter, such as  $\alpha_{2a}$ , . . .  $\alpha_{2e}$ .  
15 In addition, the  $\alpha_2$  subunit and the large fragment produced when the protein is subjected to reducing conditions appear to be glycosylated with at least N-linked sugars and do not specifically bind to the 1,4-DHPs and phenylalkylamines that specifically bind to the  $\alpha_1$  subunit. The smaller fragment, the C-terminal fragment, is referred to as the  $\delta$  subunit and  
20 includes amino acids from about 946 (as numbered in International PCT application No. WO95/04822, *e.g.*, SEQ ID No. 11 therein) through about the C-terminus. This fragment may dissociate from the remaining portion of  $\alpha_2$  when the  $\alpha_2$  subunit is exposed to reducing conditions. For purposes herein  $\alpha_2$  is also referred to as  $\alpha_2\delta$ . Thus, reference to  $\alpha_2\delta$   
25 means the  $\alpha_2$  subunit, including the C-terminal  $\delta$  portion.

As used herein, a  $\beta$  subunit is encoded by DNA disclosed, for example, in U.S. Patent No. 5,407,820, U.S. Patent No. 5,792,846 and International PCT application No. WO95/04822 or that hybridizes to the DNA provided therein under conditions of low stringency, preferably high

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modulated or altered by a 1,4-DHP. It is presently preferable to refer to calcium channels based on pharmacological characteristics and current kinetics and to avoid historical designations. Types and subtypes of  $\alpha_1$  subunits may be characterized on the basis of the effects of such modulators on the subunit or a channel containing the subunit as well as differences in currents and current kinetics produced by calcium channels containing the subunit. The  $\alpha_{1H}$  subunits may be further identified by the presence the notably long intracellular loop regions, such as between transmembrane domains I and II (e.g., nt 1506 to nt 2627 of SEQ ID No. 12), and also the loop in domain I.

In particular, nucleic acid that encodes an  $\alpha_{1H}$  subunit as used herein, will hybridize under conditions of high stringency to the nucleic acid disclosed herein as SEQ ID Nos. 12, 15 and 16, and will form a channel in a mammalian cell, such as an HEK cell, that exhibits electrophysiological and/or pharmacological properties of a LVA or T-channel. The electrophysiological properties include one or more of the following electrophysiological properties a relative conductance of  $Ba^{2+}$  of about 5 pS (picoamps) to about 9 pS, an activation time of about 2 to about 8 milliseconds, a kinetics of activation  $V_{1/2}$  value of about -60 millivolts to about -26 millivolts, an inactivation time of about 10 to about 30 milliseconds, a kinetics of inactivation  $V_{1/2}$  value of about -100 millivolts to about -500 millivolts, and a tail deactivation time of about 2 to about 12 milliseconds.

In addition, the resulting channel may have pharmacological properties, such as a relatively high degree of sensitivity to mibefradil, (1S,2S)-2-[2-[[3-(1H-benzimidazol-2-yl)propyl]methyl-amino]ethyl]-6-fluoro-1-isopropyl-1,2,3,4-tetrahydronaphthalen-2-yl methoxyacetate (Hoffman-LaRoche, Inc.) and/or a relatively high degree of resistance to the Conus

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(Madison WI), to determine a percentage of sequence identity (see, also, von Heijne, entitled "Sequence Analysis in Molecular Biology: Treasure Trove of Trivial Pursuit" Academic Press (1987) Appendix 2 (citing to UWG and DNASTar among seven commercially available software programs)).

An  $\alpha_1$  subunit may be identified by its ability to form a calcium channel. Typically,  $\alpha_1$  subunits have molecular masses greater than at least about 120 kD. Also, hydropathy plots of deduced  $\alpha_1$  subunit amino acid sequences indicate that the  $\alpha_1$  subunits contain four internal repeats, each containing six transmembrane domains. An  $\alpha_{1H}$ -subunit is identified by its pore-forming ability and also the low-voltage activation of the resulting channel.

The activity of a calcium channel may be assessed *in vitro* by methods known to those of skill in the art, including the electrophysiological and other methods described herein. Typically,  $\alpha_1$  subunits include regions with which one or more modulators of calcium channel activity, such as a 1,4-DHP or  $\omega$ -CgTx, interact directly or indirectly. Types of  $\alpha_1$  subunits may be distinguished by any method known to those of skill in the art, including on the basis of binding specificity. For example, it has been found herein that  $\alpha_{1B}$  subunits participate in the formation of channels that have previously been referred to as N-type channels,  $\alpha_{1D}$  subunits participate in the formation of channels that had previously been referred to as L-type channels,  $\alpha_{1A}$  subunits appear to participate in the formation of channels that exhibit characteristics typical of channels that had previously been designated P-type channels, and  $\alpha_{1H}$  subunits appear to participate in channels that exhibit activities associated with T-type channels. Thus, for example, the activity of channels that contain the  $\alpha_{1B}$  subunit are insensitive to 1,4-DHPs; whereas the activity of channels that contain the  $\alpha_{1D}$  subunit are

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encodes a subunit that hybridizes under at least low stringency, preferably high stringency, to nucleic acid that encodes an  $\alpha_{1H}$  subunit, and that encodes a subunit having the requisite LVA properties in assays for such activity, as those described herein. Splice variants will have  
5 varying percentages of overall homology (or identity), but will be derived from the same gene and will include regions of 100% identity.

In particular, a splice variant of any of the  $\alpha_1$  subunits (or any of the subunits particularly disclosed herein) will contain regions (at least one exon) of divergence and one or more regions (at least one exon,  
10 typically more than about 16 nucleotides, and generally substantially more) that have 100% homology with one or more of the  $\alpha_1$  subunit subtypes provided herein, and will also contain a region that has substantially less homology, since it is derived from a different exon. It is well within the skill of those in this art to identify exons and splice  
15 variants. Thus, for example, an  $\alpha_{1H}$  subunit will be readily identifiable, because it will share at least about 40% protein homology with one of the  $\alpha_{1H}$  subunits disclosed herein, and will include at least one region (one exon) that is 100% homologous. It will also have activity, as discussed below, that indicates that it is an LVA  $\alpha_1$  subunit.

20 It is noted herein, that identity and homology refer to the percentage of amino acids when proteins are compared or nucleotides when nucleic acids are compared that are shared. Numerous computer programs for determining identity are available. In all instances, intended gap penalties and other parameters are the defaults set by the  
25 manufacturer. Although not really needed when there is a high (90% or greater) degree of identity between sequences such programs include, but are not limited to commercially available sequence alignment programs, such as the DNASTar "MegAlign" program (Madison, WI) and the University of Wisconsin Genetics Computer Group (UWG) "Gap" program

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- (a) a sequence of nucleotides that encodes a human calcium  $\alpha_{1H}$  channel subunit and includes a sequence of nucleotides set forth in any of the SEQ ID's herein (i.e., SEQ ID Nos. 12, 15 and 16) that encodes such subunit;
- 5 (b) a sequence of nucleotides that encodes the subunit and hybridizes under conditions of high stringency to DNA that is complementary to an mRNA transcript present in a human cell that encodes a LVA subunit, particularly an  $\alpha_{1H}$ -subunit;
- (c) a sequence of nucleotides that encodes the subunit that  
10 includes a sequence of amino acids encoded by any of SEQ ID Nos. 12-16; and
- (d) a sequence of nucleotides that encodes a subunit that includes a sequence of amino acids encoded by a sequence  
15 of nucleotides that encodes such subunit and hybridizes under conditions of high stringency to DNA that is complementary to an mRNA transcript present in a human cell that encodes the subunit that includes a sequence of nucleotides set forth in any of SEQ ID Nos. 12-16.

As used herein, the  $\alpha_1$  subunit types, encoded by different genes,  
20 are designated as type  $\alpha_{1A}$ ,  $\alpha_{1B}$ ,  $\alpha_{1C}$ ,  $\alpha_{1D}$ ,  $\alpha_{1E}$  and  $\alpha_{1H}$ . These types have also been referred to as VDCC IV for  $\alpha_{1B}$ , VDCC II for  $\alpha_{1C}$  and VDCC III for  $\alpha_{1D}$ . Subunit subtypes, which are splice variants, are referred to, for example as  $\alpha_{1H-1}$ ,  $\alpha_{1H-2}$ ,  $\alpha_{1B-1}$ ,  $\alpha_{1B-2}$ ,  $\alpha_{1C-1}$  etc.

Thus, as used herein, nucleic acid (DNA or RNA) encoding the  $\alpha_1$   
25 subunit refers to nucleic acid that hybridizes to the DNA provided herein under conditions of at least low stringency, typically high stringency, or encodes a subunit that has at least about 40% homology to protein encoded by DNA disclosed herein that encodes the specified  $\alpha_1$  subunit of a human calcium channel. In the case of LVA channels, nucleic acid that



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DNA provided herein and the protein encoded by such DNA exhibits additional identifying characteristics, such as function or molecular weight. In particular, reference to an  $\alpha_{1H}$  subunit refers to subunits that can be isolated from nucleic acid libraries from any desired source using  
5 the nucleic acid disclosed herein as a probe. The encoded subunit is characterized by the presence of the notably long intracellular loop between transmembrane domains I and II, and/or properties ascribed to T-type or LVA type channels.

It is understood that subunits that are encoded by transcripts that  
10 represent splice variants of the disclosed subunits or other such subunits may exhibit less than 40% overall homology to any single subunit, but will include regions of such homology to one or more such subunits. It is also understood that 40% homology refers to proteins that share approximately 40% of their amino acids in common or that share  
15 somewhat less, but include conservative amino acid substitutions, whereby the activity of the protein is not substantially altered.

The subunits and DNA fragments encoding such subunits are provided herein or known to those of skill in the art (see, published International PCT application Nos. WO89/09834, WO93/04083,  
20 WO95/04822, U.S. Patent Nos. 5,792,846, 5,726,035, 5,407,820, 5,686,241, 5,618,720, 5,710,250, 5,429,921, 5,429,921 and 5,386,025) include any  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$  or  $\gamma$  subunits of a human calcium channel.

Nucleic acid encoding LVA subunits, particularly  $\alpha_{1H}$  subunits of  
25 human and other animal calcium channels, are provided herein. In particular, such DNA fragments include any isolated DNA fragment that (encodes a subunit of a human calcium channel, that (1) contains a sequence of nucleotides that encodes the subunit, and (2) is selected from among:

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FIGURE 3 schematically depicts features of the  $\alpha_{1H-1}$  subunit and shows amino acid sequence alignment of human  $\alpha_{1H}$  with  $\alpha_{1D}$  and  $\alpha_{1E}$  in each of the four pore regions; \* indicates residues involved in ion selectivity in each of the four pore regions; the unusually large loop in the LVA-associated  $\alpha_{1H}$  subunits between transmembrane domains I and II.

FIGURE 4A shows the tail currents elicited by repolarization to -90 mV following 10 ms step depolarizations between -80 and -10 mV. For tail current measurements the digitization/filter rates were 50/16 kHz. Tail current decay was fitted to a bi-exponential function of the form  $I = A_0 + A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$ . The bi-exponential decay profile of the tail current was observed in every cell examined ( $n = 12$ ). FIGURES 4B and 4C show the voltage-dependence of the time constants  $\tau_1$  and  $\tau_2$  for current deactivation (FIGURE 4B) and the current fractions  $A_1$  and  $A_2$  (FIGURE 4C).

## 15 DETAILED DESCRIPTION OF THE INVENTION

### Definitions:

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents and publications referred to herein are incorporated by reference herein.

Reference to each of the calcium channel subunits includes the subunits that are specifically disclosed herein and human calcium channel subunits encoded by nucleic acid that can be isolated by using the nucleic acid disclosed as probes and screening an appropriate human cDNA or genomic library under at least low stringency, preferably high stringency. Such DNA also includes DNA that encodes proteins that have about 40% homology, typically at least about 90% sequence identity taking into account gaps) to any of the subunits proteins described herein or DNA or RNA that hybridizes under conditions of at least low stringency to the

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function, such altered amino acid sequences, altered pharmacological profiles and altered electrophysiological profiles compared to normal or wild-type channels. Such methods typically can employ antibodies specific for the altered channel or nucleic acid probes to detect altered  
 5 genes or transcripts.

#### DESCRIPTION OF THE FIGURES

FIGURE 1 shows the voltage-dependence of activation ( $m_{\infty}$ ) and steady-state inactivation ( $h$ ) of human  $\alpha_{1H}$  calcium channels expressed transiently in HEK cells. Voltage-dependence of activation ( $m_{\infty}$ ) was  
 10 determined from tail current analysis. Tail currents were normalized with respect to the maximum peak tail current obtained at +60 mV and were plotted (open symbols, mean  $\pm$  SEM;  $n = 11$ ) vs. test potential. Data were fitted by the sum of two Boltzman function  $m_{\infty} = F_A * [1 + \exp(-(V_{test} - V_{1/2,A})/k_A)]^{-1} + F_B * [1 + \exp(-(V_{test} - V_{1/2,B})/k_B)]^{-1}$ ,  $F_A = 0.67$ ,  $V_{1/2,A} = -$   
 15 21.5mV,  $k_A = 7.5$ ,  $F_B = 0.33$ ,  $V_{1/2,B} = 25.5$  mV,  $k_B = 14.7$ . Steady-state inactivation ( $h_{\infty}$ ) was determined from a holding potential of -100 mV by a test pulse to -20 mV (p1), followed by a 20 second prepulse from -100 mV to -10 mV in 5 mV decrements (pHold) preceding a second test pulse to -20 mV (p2). Normalized current amplitudes were plotted (closed  
 20 symbols, mean  $\pm$  SEM;  $n = 9$ ) vs. holding potential. Data were fitted by a Boltzman function  $h_{\infty} = [1 + \exp((V_{hold} - V_{1/2})/k)]^{-1}$ ,  $V_{1/2} = -63.9$  mV,  $k = 3.9$  mV.

FIGURE 2 shows the kinetics of activation (FIGURE 2A) and inactivation (FIGURE 2B) of human  $\alpha_{1H}$  ( $\alpha_{1H.1}$ ) calcium channels; kinetics of  
 25 activation and inactivation were determined from current traces by fitting an exponential function to rising (FIG. 2A) or declining (FIG. 2B) phase of the current (the voltage-dependence for activation and inactivation follows approximately an exponential function).

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and channels can be isolated from a eukaryotic cell transfected with nucleic acid that encodes the subunit.

In another embodiment, immunoglobulins or antibodies obtained from the serum of an animal immunized with a substantially pure  
5 preparation of a human calcium channel, human calcium channel subunit or epitope-containing fragment of a human calcium subunit are provided. Monoclonal antibodies produced using a human calcium channel, human calcium channel subunit or epitope-containing fragment thereof as an  
10 immunogen are also provided. *E. coli* fusion proteins including a fragment of a human calcium channel subunit may also be used as immunogen. Such fusion proteins may contain a bacterial protein or portion thereof, such as the *E. coli* TrpE protein, fused to a calcium channel subunit peptide. The immunoglobulins that are produced using the calcium  
15 channel subunits or purified calcium channels as immunogens have, among other properties, the ability to specifically and preferentially bind to and/or cause the immunoprecipitation of a human calcium channel or a subunit thereof which may be present in a biological sample or a solution derived from such a biological sample. Such antibodies may also be  
20 used to selectively isolate cells that express calcium channels that contain the subunit for which the antibodies are specific.

Methods for modulating the activity of ion channels by contacting the calcium channels with an effective amount of the above-described antibodies are also provided.

Thus, assays for identifying compounds that modulate the activity  
25 of LVA calcium channels, particularly T-type channels are provided as well as compounds identified by the methods.

Also provided are methods for diagnosing LVA calcium channel-mediated, particularly T-type channel-mediated, disorders. Methods of diagnosis will involve detection of aberrant channel expression or

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identify compounds useful in regulating vascular smooth muscle tone, either vasodilating or vasoconstricting in: (a) treatments for reestablishing blood pressure control, e.g., following traumatic injury, surgery or cardiopulmonary bypass, and in prophylactic treatments designed to

5 minimize cardiovascular effects of anaesthetic drugs; (b) treatments for improving vascular reflexes and blood pressure control by the autonomic nervous system; for identifying compounds useful in treating urological disorders: (a) treating and restoring renal function following surgery, traumatic injury, uremia and adverse drug reactions; (b) treating bladder

10 dysfunctions; and (c) uremic neuronal toxicity and hypotension in patients on hemodialysis; reproductive disorders, for identifying compounds useful in treating: (a) disorders of sexual function including impotence; (b) alcoholic impotence (under autonomic control that may be subject to T-channel controls); hepatic disorders for identifying compounds useful in

15 treating and reducing neuronal toxicity and autonomic nervous system damage resulting from acute over-consumption of alcohol; neurologic disorders for identifying compounds useful in treating: (a) epilepsy and diencephalic epilepsy; (b) Parkinson's disease; (c) aberrant temperature control, such as, abnormalities of shivering and sweat gland secretion and

20 peripheral vascular blood supply; (d) aberrant pituitary and hypothalamic functions including abnormal secretion of noradrenaline, dopamine and other hormones; for respiratory such as in treating abnormal respiration, e.g., post-surgical complications of anesthetics; and endocrine disorders, for identifying compounds useful in treating aberrant secretion of

25 hormones including e.g., possible treatments for overproduction of insulin, thyroxin, adrenalin, and other hormonal imbalances.

Purified human  $\alpha_{1H}$  calcium channel subunits and purified human calcium channels containing such subunits are provided. The subunits

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disorders, disease and conditions. Identified compounds will be candidates for use in the treatment of disorders and conditions associated with T-channel activity. Such activities include, but are not limited to, those involving role in muscle excitability, secretion and pacemaker

5 activity,  $\text{Ca}^{2+}$  dependent burst firing, neuronal oscillations, and potentiation of synaptic signals, for improving arterial compliance in systolic hypertension, or improving vascular tone, such as by decreasing vascular welling, in peripheral circulatory disease, and others. Other disorders include, but are not limited to hypertension, cardiovascular

10 disorders, including but not limited to: myocardial infarct, cardiac arrhythmia, heart failure and angina pectoris; neurological disorders, such as schizophrenia, epilepsy and depression, peripheral muscle disorders, respiratory disorders and endocrine disorders.

In particular, cells that express LVA channels, such as the  $\alpha_{1H}$

15 subunits, are useful for identifying compounds that are candidates for treatment of disorders associated with conduction tissues, such as atrial pacemaker cells, Purkinje fibers, and also coronary smooth muscles. Such disorders include, but are not limited to, compounds useful for treatment of cardiovascular, such as angina, vascular, such as

20 hypertension, and urologic, hepatic, reproductive, adjunctive therapies for reestablishing normal heart rate and cardiac output following traumatic injury, heart attack and other cardiac injuries; treatments of myocardial infarct (MI), post-MI and in an acute setting. Other compounds that interact with LVA, particularly T-type, calcium channels, may be effective

25 for increasing cardiac contractile force, such as measured by left ventricular end diastolic pressure, and without changing blood pressure or heart rate. In an acute other compounds may be effective to decrease formation of scar tissue, such as that measured by collagen deposition or septal thickness, and without cardiodepressant effects. The assays may

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more preferably an  $\alpha_{1H}$ -subunit encoded by heterologous DNA, and also contain nucleic acid encoding a reporter gene construct containing a reporter gene in operative linkage with one or more transcriptional control elements that is regulated by a calcium channel. The assays are effected

5 by comparing the difference in the amount of transcription of a the reporter gene in the cells provided herein in the presence of the compound with the amount of transcription in the absence of the compound, or with the amount of transcription in the absence of the heterologous calcium channel, whereby compounds that modulate the

10 activity of the heterologous calcium channel in the cell are identified. The reporter gene is any such gene known to those of skill in the art, including, but not limited to the gene encoding bacterial chloramphenicol acetyltransferase, the gene encoding firefly luciferase, the gene encoding bacterial luciferase, the gene encoding  $\beta$ -galactosidase or the gene

15 encoding alkaline phosphatase, and the transcriptional control element is any such element known to those of skill in the art, including, but not limited to serum responsive elements, cyclic adenosine monophosphate responsive elements, the *c-fos* gene promoter, the vasoactive intestinal peptide gene promoter, the somatostatin gene promoter, the

20 proenkephalin promoter, the phosphoenolpyruvate carboxykinase gene promoter or the nerve growth factor-1 A gene promoter and elements responsive to intracellular calcium ion levels.

Other assays in which receptor activity in response to test compounds is measured may also be practiced with the cells provided

25 herein (see, e.g., U.S. Patent No. 5,670,113).

Because T-type channels appear to be associated with a variety of key functions, cells that express T-channels and assays using such cells will be useful for identification of compounds for treatment of a variety of

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activities such as heart rate, arterial and venous smooth muscle innervation and tone, pulmonary rate and other fundamental processes, assays designed to assess such activities and assays the identify modulators of these activities provide a means to understand fundamental physiological processes and also a means to identify new drug candidates for an array of disorders.

Assays that use the eukaryotic cells for identifying compounds that modulate calcium channel activity are also provided. In practicing these assays the eukaryotic cell that expresses a heterologous calcium channel, containing at least one subunit encoded by the DNA provided herein, is in a solution containing a test compound and a calcium channel selective ion, the cell membrane is depolarized, and current flowing into the cell is detected. If the test compound is one that modulates calcium channel activity, the current that is detected is different from that produced by depolarizing the same or a substantially identical cell in the presence of the same calcium channel-selective ion but in the absence of the compound. In preferred embodiments, prior to the depolarization step, the cell is maintained at a holding potential which substantially inactivates calcium channels which are endogenous to the cell. Also in preferred embodiments, the cells are mammalian cells, most preferably HEK cells, or amphibian oocytes.

Cells that express T-channels or LVA channels may be used in assays that screen for compounds that have activity as modulators, particularly antagonists, of the activity of these channels.

Transcription based assays for identifying compounds that modulate the activity of calcium channels (see, U.S. Patent Nos. 5,436,128 and 5,401,629), particularly calcium channels that contain an  $\alpha_{1H}$  subunit are provided. These assays use cells that express calcium channels, particularly calcium channels containing an  $\alpha_{1H}$ -subunit, and



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produced by transfection with DNA encoding one or more of the subunits or are injected with RNA transcripts of DNA encoding one or more of the calcium channel subunits. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or  
5 transient expression of the subunit-encoding DNA. Vectors containing DNA encoding human calcium channel subunits are also provided.

The eukaryotic cells that express heterologous calcium channels may be used in assays for calcium channel function or, in the case of cells transformed with fewer subunit-encoding nucleic acids than  
10 necessary to constitute a functional recombinant human calcium channel, such cells may be used to assess the effects of additional subunits on calcium channel activity. The additional subunits can be provided by subsequently transfecting such a cell with one or more DNA clones or RNA transcripts encoding human calcium channel subunits.

15 The recombinant eukaryotic cells that express membrane spanning heterologous calcium channels may be used in methods for identifying compounds that modulate calcium channel activity. In particular, the cells are used in assays that identify agonists and antagonists of calcium channel activity in humans and/or assessing the contribution of the  
20 various calcium channel subunits to the transport and regulation of transport of calcium ions. Because the cells constitute homogeneous populations of calcium channels, they provide a means to identify agonists or antagonists of calcium channel activity that are specific for each such population.

25 The cells provided herein may be used to assess T-type channel function and tissue distribution and to identify compounds that modulate the activity of T-type channels. Because T-type channels are operative in neurons in the thalamus, hypothalamus, and brain stem, and may be involved in autonomic nervous functions, in regulation of cardiovascular

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nucleic acid encoding an  $\alpha_{1H}$  are of particular interest herein. In other embodiments, the cells contain DNA or RNA encoding additional heterologous subunits, including an  $\alpha_2\delta$ . The cells may also include nucleic acid encoding a  $\beta$  subunit and/or a  $\gamma$  subunit. In such

5   embodiments, eukaryotic cells stably or transiently transfected with any combination of one, two, three or four of the subunit-encoding DNA clones, such as DNA encoding any of  $\alpha_1$ ,  $\alpha_1 + \beta$ ,  $\alpha_1 + \beta + \alpha_2$ , are provided. The eukaryotic cells provided herein contain heterologous nucleic acid that encodes an  $\alpha_1$  subunit and optionally a heterologous  $\alpha_2$ -

10   subunit and/or a  $\beta$  subunit and/or  $\gamma$  subunit.

In preferred embodiments, the cells express such heterologous calcium channel subunits and include one or more of the subunits in membrane-spanning heterologous calcium channels. In more preferred embodiments, the eukaryotic cells express functional, heterologous

15   calcium channels that are capable of gating the passage of calcium channel-selective ions and/or binding compounds that, at physiological concentrations, modulate the activity of the heterologous calcium channel. In certain embodiments, the heterologous calcium channels include at least one heterologous calcium channel subunit. In most

20   preferred embodiments, the calcium channels that are expressed on the surface of the eukaryotic cells are composed substantially or entirely of subunits encoded by the heterologous DNA or RNA. In preferred embodiments, the heterologous calcium channels of such cells are distinguishable from any endogenous calcium channels of the host cell.

25   Such cells provide a means to obtain homogeneous populations of calcium channels. Typically, the cells contain the selected calcium channel as the only heterologous ion channel expressed by the cell.

In certain embodiments the recombinant eukaryotic cells that contain the heterologous DNA encoding the calcium channel subunits are

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also slightly larger than the HVA  $\alpha_1$  subunits (186 nt compared to 159-165 nt).

Nucleic acid probes, which can be labeled for detection, containing at least about 14, preferably 16, or, if desired, 20 or 30 or more, 5 contiguous nucleotides of  $\alpha_{1H}$ -encoding nucleic acid are provided. Methods using the probes for the isolation and cloning of calcium channel subunit-encoding DNA, including splice variants within tissues and inter-tissue variants are also provided. Particularly preferred regions from which to construct probes for the isolation of DNA encoding a human  $\alpha_{1H}$  10 subunit include the nucleic acid sequence encoding the notably long intracellular loop located between transmembrane Domains I and II (e.g., nt 1506 to nt 2627 of SEQ ID Nos. 12 and 15). Probes for isolating DNA encoding a human  $\alpha_{1H}$  subunit are preferably 14 or 16 contiguous nucleotides in length. In some instances, probes of 30 or 50 nucleotides 15 are used and in other instances probes between 50 to 100 nucleotides are used.

Eukaryotic cells containing heterologous DNA encoding one or more calcium channel subunits, particularly human calcium channel subunits, or containing RNA transcripts of DNA clones encoding one or 20 more of the subunits are provided. A single  $\alpha_{1H}$  subunit can form a channel. The requisite combination of subunits for formation of active channels in selected cells, however, can be determined empirically using the methods herein. For example, if a selected  $\alpha_1$  subtype or variant does not form an active channel in a selected cell line, an additional subunit or 25 subunits can be added until an active channel is formed. Other subunits can be added to assess the effects of such addition.

In preferred embodiments, the cells contain DNA or RNA encoding an  $\alpha_1$  subunit, preferably an  $\alpha_{1H}$  subunit of an animal, preferably of a mammalian calcium channel. Embodiments in which the cells contain

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Also included are any subunits that are encoded by nucleic acid containing nucleotides nt 1506 to nt 2627 of SEQ ID No. 12 or 15 or subunits that are encoded by nucleic acid that hybridizes, preferably under conditions of high stringency, to a probe derived from this region  
5 and that encodes a T-channel, which can be identified using methods herein.

The  $\alpha_{1H}$  subunit differs from the  $\alpha_{1A}$ - $\alpha_{1E}$  calcium channel subunits in a number of aspects. First, the intracellular loop positioned between transmembrane Domains I and II is considerably longer than HVA calcium  
10 channels. For instance, as exemplified in SEQ ID Nos. 12 and 15 and described below, the intracellular loop between Domains I and II is greater than 1,100 nt (1122 nt), whereas the corresponding region in HVA calcium channels ranges from 351 to 381 nt in length. Thus, the intracellular loop of  $\alpha_{1H}$  contains approximately 370 additional amino acid  
15 residues (aa 420 to aa 794 of SEQ ID No. 12) not found in HVA calcium channel  $\alpha_1$  subunits. In addition, the encoded amino acid sequence of this loop region is highly proline rich and contains a poly-HIS region of 9 consecutive histidine residues.

Other distinguishing features of the  $\alpha_{1H}$  subunit, include the  
20 absence of amino acid residues in the intracellular loop between transmembrane Domains I and II that are known to be critical (e.g., see De Waard *et al.* (1996) FEBS Letters 380:272-276; Pragnell *et al.* (1994) Nature 368:67-70) for the interaction between an  $\alpha_1$  subunit and a  $\beta$  subunit. The  $\alpha_{1H}$  subunit also contains a notably large extracellular loop in  
25 Domain I between IS5 and IS6. The HVA  $\alpha_1$  calcium channel subunits provided herein contain 249-270 nucleotide residues in this loop. In contrast, the human  $\alpha_{1H}$  subunit contains 426 nucleotide residues in this loop. The intracellular loop between transmembrane Domains III and IV is

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DNA encoding these  $\alpha_1$  subunits of a animal channels, and RNA, encoding such subunits, made upon transcription of such DNA are provided. In particular, nucleic acid that encodes T-type calcium channels, designated  $\alpha_{1H}$ -subunits (designated  $\alpha_{1F}$  in the priority document  
5 U.S. application Serial No. 08/984,709) of a calcium channel, particularly an animal calcium channel and more particularly a mammalian calcium channel is provided.

Of particular interest herein is the nucleic acid that encodes the  $\alpha_{1H}$  subunits of calcium channels, particularly mammalian calcium channels.  
10 Nucleic acid encoding exemplary  $\alpha_{1H}$  subunits are provided. Nucleic acid encoding two splice variants, designated  $\alpha_{1H-1}$  and  $\alpha_{1H-2}$ , from human calcium channels is provided. The nucleic acid sequences and encoded amino acids of the exemplified subunits are set forth in SEQ ID Nos. 12 ( $\alpha_{1H-1}$ ), 15 ( $\alpha_{1H-1}$ ) and 16 ( $\alpha_{1H-2}$ ). SEQ ID NOs. 12 and 15 differ only in that  
15 in amino acid 2230 (bases 6983-6985) is Asp (GAC) in the SEQ ID No. 15 and Glu (GAA) in SEQ ID No. 12.

This nucleic acid can be used to isolate variants, including additional splice variants of the nucleic acid encoding  $\alpha_{1H}$  subunits, allelic variants and  $\alpha_{1H}$  subunits from other animals, particularly mammals. Such  
20 nucleic acid includes DNA encoding an  $\alpha_{1H-1}$  subunit that has substantially the same sequence of amino acids as encoded by the DNA set forth in SEQ ID Nos. 12 and 15. This nucleic acid can also be used to isolate DNA encoding  $\alpha_{1H}$  subunits from other species, particularly other mammals.

25 Also provided is nucleic acid that encodes a second splice variant, designated  $\alpha_{1H-2}$ , is provided. The nucleic acid sequence of this variant, differs from  $\alpha_{1H-1}$  in having a 957 nucleotide deletion, resulting in loss of 319 amino acids (corresponding to amino acids 470-788 of  $\alpha_{1H-1}$ ).

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structure of calcium channels that generate the various LVA currents is unknown. None of the  $\alpha_1$  subunits previously cloned appear to have all properties that have been ascribed to the low voltage-activated T-type (or LVA) channels.

5           Therefore, it is an object herein, to provide nucleic acid encoding specific calcium channel subunits that have structural and functional properties that differ from the HVA type channels. It is also an object herein to provide nucleic acid encoding channels that have activities that have been ascribed to T-type channels and to provide eukaryotic cells  
10 bearing recombinant tissue-specific or subtype-specific calcium channels. It is also an object to provide assays for identification of potentially therapeutic compounds that act as modulators of calcium channel activity, particularly those specific for channels that exhibit properties of human T-type channels and other types of channels.

## 15   SUMMARY OF THE INVENTION

Isolated and purified nucleic acid fragments that encode calcium channel subunits are provided. The subunits form low-voltage activated (LVA) channels, particularly channels that have properties associated with T-type channels. The subunits and results provided herein, provide a  
20 family of  $\alpha_1$  subunits corresponding to LVA, or T-type, channels. Channels that contain these subunits have ability to open at low potential difference, but stay open for only moderate time periods. These channels are located in critical physiologic locations, including neurons in the thalamus, hypothalamus, and brain stem, and consequently may be  
25 involved in autonomic nervous functions, perhaps involved in regulation of cardiovascular activities such as heart rate, arterial and venous smooth muscle innervation and tone, pulmonary rate and other critical physiologic activities.

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specific, increases the current generated by any  $\alpha_1$  subunit and potentiates the stimulatory response of  $\beta$  subunits.

#### T-type or LVA channels

Little is known about the channels that have been designated T-channels or LVA (low voltage activated) channels. Low-voltage activated (LVA), i.e., T-type, calcium channels are reportedly found in a variety of cell types. Low-voltage activated (LVA) or T-type calcium channels are also widely distributed in the central and peripheral nervous system and apparently involved in an extensive array of different neuronal processes.

In general it is believed that T-type currents do not differ fundamentally from other  $\text{Ca}^{2+}$  currents. Like HVA channels, T-type channels are selectively permeable to divalent cations, as long as a minimal concentration of divalent cations is present in the external medium. For LVA (or T-type) currents, this minimal  $\text{Ca}^{2+}$  concentration is about 25  $\mu\text{M}$ , and for HVA currents it is about 1  $\mu\text{M}$ . T-type current is reported to saturate with a  $K_d$  of about 10 mM  $\text{Ca}^{2+}$ , which is similar to that reported for HVA currents. The channels, however, appear to exhibit certain differences. They differ in their relative permeability to divalent cations. In general, HVA channels are more permeable to  $\text{Ba}^{2+}$  than to  $\text{Ca}^{2+}$ ; T-type are equally or slightly less permeable to  $\text{Ba}^{2+}$  than to  $\text{Ca}^{2+}$ . T-type channels also are believed to exhibit slower activation/inactivation and deactivation kinetics and have been reported to exhibit relatively higher sensitivity to  $\text{Ni}^{2+}$ . This type of channel is activated near the resting potential of the membrane, and is believed to be responsible for the generation of repetitive firing activity or intrinsic neuronal oscillations and for  $\text{Ca}^{2+}$  entry accompanying the spike activity (see, e.g., Huguenard (1996) Annual Rev. Physiol. 58:329-348). Recent data suggests that  $\beta$ -subunits identified to date may not be a constitutive T-type channel subunit (see, Lambert et al. (1997) J. Neurosci. 17:6621-6625). The

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muscle, lung, smooth muscle and brain, (see, *e.g.*, Bean, B.P.(1989) *Ann. Rev. Physiol.* 51:367-384 and Hess, P. (1990) *Ann. Rev. Neurosci.* 56:337). The different types of calcium channels have been broadly categorized into four classes, L-, T-, N-, P-, Q and R-type, distinguished  
5 by current kinetics, holding potential sensitivity and sensitivity to calcium channel agonists and antagonists. The primary determinant of diversity among calcium channels is the nature of the pore-forming  $\alpha_1$  subunit. Nucleic acid encoding numerous  $\alpha_1$  subunits has been cloned and the encoded subunits expressed. Correlations between  $\alpha_1$  subunits and the  
10 operationally defined  $\text{Ca}^{2+}$  currents have been established. Six gene products  $\alpha_{1A}$ - $\alpha_{1E}$  and  $\alpha_{1S}$  participate in the formation of high-voltage activated channels, which include the L, N, P, Q and R-type channels.

DNA encoding human  $\alpha_1$ -subunits, including  $\alpha_{1A}$ -,  $\alpha_{1B}$ -,  $\alpha_{1C}$ -,  $\alpha_{1D}$ - and  $\alpha_{1E}$  subunits and splice variants thereof has been described (see, *e.g.*,  
15 U.S. Patent No. 5,429,921, U.S. Patent No. 5,846,756, U.S. Patent No. 5,851,824, published International PCT application No. PCT/US92/06903, and published International PCT application No. PCT/US94/09230). These subunits appear to participate in formation of high voltage calcium (HVA) channels, which in addition to one of these  
20  $\alpha_1$ -subunits, includes a  $\beta$  subunit and an  $\alpha_2$ -subunit, including  $\delta$ , which is linked to  $\alpha_2$  by a disulfide bridge and arises from the same precursor. The distinct biophysical and pharmacological properties of each channel derive primarily from the  $\alpha_1$ -subunit, but are modulated by the ancillary subunits, principally the  $\beta$  subunits associated with the channel.  $\beta$ -subunits have  
25 been shown to increase the peak current amplitude, to shift activation/inactivation curves toward more hyperpolarized potentials and to alter kinetics of activation and inactivation (see, *e.g.*, Lambert *et al.* (1997) *J. Neurosci.* 17:6621-6625). The  $\alpha_2\delta$  subunit, which is tissue-



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containing as one component the respective  $\alpha_1$  subunits *in vivo* is unclear. In order to completely and accurately characterize and evaluate different calcium channel types, however, it is essential to examine the functional properties of recombinant channels containing all of the subunits as found  
5 *in vivo*.

In order to conduct this examination and to fully understand calcium channel structure and function, it is critical to identify and characterize as many calcium channel subunits as possible. Also in order to prepare recombinant cells for use in identifying compounds that  
10 interact with calcium channels, it is necessary to be able to produce cells that express uniform populations of calcium channels containing defined subunits.

An understanding of the pharmacology of compounds that interact with calcium channels in other organ systems, such as the CNS, may aid  
15 in the rational design of compounds that specifically interact with subtypes of human calcium channels to have desired therapeutic effects, such as in the treatment of neurodegenerative and cardiovascular disorders. Such understanding and the ability to rationally design therapeutically effective compounds, however, have been hampered by  
20 an inability to independently determine the types of human calcium channels and the molecular nature of individual subtypes, particularly in the CNS, and by the unavailability of pure preparations of specific channel subtypes to use for evaluation of the specificity of calcium channel-affecting compounds. Thus, identification of DNA encoding human  
25 calcium channel subunits and the use of such DNA for expression of calcium channel subunits and functional calcium channels would aid in screening and designing therapeutically effective compounds.

Multiple types of calcium channels have been identified in mammalian cells from various tissues, including skeletal muscle, cardiac

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apparent molecular weight of 30-33 kD, as determined by SDS-PAGE analysis.

In order to study calcium channel structure and function, large amounts of pure channel protein are needed. Because of the complex nature of these multisubunit proteins, the varying concentrations of calcium channels in tissue sources of the protein, the presence of mixed populations of calcium channels in tissues, difficulties in obtaining tissues of interest, and the modifications of the native protein that can occur during the isolation procedure, it is extremely difficult to obtain large amounts of highly purified, completely intact calcium channel protein.

Because calcium channels are present in various tissues and have a central role in regulating intracellular calcium ion concentrations, they are implicated in a number of vital processes in animals, including neurotransmitter release, muscle contraction, pacemaker activity, and secretion of hormones and other substances. These processes appear to be involved in numerous human disorders, such as central nervous system disorders and cardiovascular diseases. Calcium channels, thus, are also implicated in numerous disorders. A number of compounds useful for treating various cardiovascular diseases in animals, including humans, are thought to exert their beneficial effects by modulating functions of voltage-dependent calcium channels present in cardiac and/or vascular smooth muscle. Many of these compounds bind to calcium channels and block, or reduce the rate of, influx of  $\text{Ca}^{2+}$  into the cells in response to depolarization of the cell membrane.

The results of studies of recombinant expression of rabbit calcium channel  $\alpha_1$  subunit-encoding cDNA clones and transcripts of the cDNA clones indicate that the  $\alpha_1$  subunit forms the pore through which calcium enters cells. The relevance of the barium currents generated in these recombinant cells to the actual current generated by calcium channels

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The most common type of calcium channel is voltage dependent. All "excitable" cells in animals, such as neurons of the central nervous system (CNS), peripheral nerve cells and muscle cells, including those of skeletal muscles, cardiac muscles, and venous and arterial smooth  
5 muscles, have voltage-dependent calcium channels (VGCCs). "Opening" of a voltage-dependent channel to allow an influx of  $\text{Ca}^{2+}$  ions into the cells requires a depolarization to a certain level of the potential difference between the inside of the cell bearing the channel and the extracellular environment bathing the cell. The rate of influx of  $\text{Ca}^{2+}$  into the cell  
10 depends on this potential difference.

Calcium channels are multisubunit proteins that contain two large subunits, designated  $\alpha_1$  and  $\alpha_2$ , which have molecular weights between about 130 and about 200 kilodaltons ("kD"), and one to three different smaller subunits of less than about 60 kD in molecular weight. At least  
15 one of the larger subunits and possibly some of the smaller subunits are glycosylated. Some of the subunits are capable of being phosphorylated. The  $\alpha_1$  subunit has a molecular weight of about 150 to about 170 kD when analyzed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) after isolation from mammalian muscle tissue and  
20 has specific binding sites for various 1,4-dihydropyridines (DHPs) and phenylalkylamines. Under non-reducing conditions (in the presence of N-ethylmaleimide), the  $\alpha_2$  subunit migrates in SDS-PAGE as a band corresponding to a molecular weight of about 160-190 kD. Upon reduction, a large fragment and smaller fragments are released. The  $\beta$   
25 subunit of the rabbit skeletal muscle calcium channel is a phosphorylated protein that has a molecular weight of 52-65 kD as determined by SDS-PAGE analysis. This subunit is insensitive to reducing conditions. The  $\gamma$  subunit of the calcium channel appears to be a glycoprotein with an

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This application is also related to U.S. application Serial No. 08/311,363, filed September 23, 1994, which is a continuation of allowed U.S. application Serial No. 07/745,206, filed August 15, 1991.

This application is also related to allowed U.S. application Serial  
5 No. 08/193,078, now U.S. Patent No. 5,846,756, filed February 7, 1994, which is the National Stage of International PCT Application No. PCT/US92/06903, published as International PCT application No. WO93/04083, filed August 14, 1992 and which is a continuation-in-part of U.S. application Serial Nos. 07/868,354, 07/745,206, 07/603,751,  
10 07/176,899, 07/620,250, filed November 30, 1990, now abandoned, and 07/482,384, now U.S. Patent No. 5,386,025, filed February 2, 1990.

This application is also related to allowed U.S. application Serial No. 08/336,257, now U.S. Patent No. 5,726,035, filed November 7,  
15 1994, which is a continuation of 07/482,384, now U.S. Patent No. 5,386,025, filed February 2, 1990.

Where permitted, the subject matter of each of the above-noted U.S. applications, patents and International PCT applications is incorporated herein in its entirety.

## 20 TECHNICAL FIELD

The present invention relates to molecular biology and pharmacology. More particularly, the invention relates to calcium channel compositions and methods of making and using the same.

## BACKGROUND OF THE INVENTION

25 Calcium channels are membrane-spanning, multi-subunit proteins that allow controlled entry of  $\text{Ca}^{2+}$  ions into cells from the extracellular fluid. Cells throughout the animal kingdom, and at least some bacterial, fungal and plant cells, possess one or more types of calcium channel.

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which is a continuation-in-part of U.S. application Serial No. 07/176,899, filed April 4, 1988, now abandoned.

This application is also related to U.S. application Serial No. 08/884,599, filed June 27, 1997, which is a continuation of U.S.  
5 application Serial No. 08/314,083.

This application is also related to U.S. application Serial No. 08/290,012, filed August 11, 1994, now abandoned, which corresponds to published International PCT application No. WO95/04822, which is a continuation-in-part of allowed U.S. application Serial No. 08/149,097,  
10 filed November 5, 1993, and a continuation-in-part of United States Application Serial No. 08/105,536, filed August 11, 1993. United States Application Serial No. 08/149,097 is a continuation-in-part of United States Application Serial No. 08/105,536, which is a continuation-in-part of the above-mentioned United States Application Serial No. 07/603,751,  
15 filed November 8, 1990.

This application is also related to allowed U.S. application Serial No. 08/223,305, filed April 4, 1994, now U.S. Patent No. 5,851,824, which is a continuation of U.S. application Serial No. 07/868,354, now abandoned, which is a continuation-in-part of U.S. application Serial No.  
20 07/745,206, filed August 15, 1991, now U.S. Patent No. 5,429,921, which is a continuation-in-part of the above-mentioned United States Application Serial No. 07/603,751, filed November 8, 1990, and a continuation-in-part of U.S. application Serial No. 07/620,250, filed November 30, 1990, now abandoned. This application is also related to  
25 allowed application U.S. application Serial No. 08/455,543, filed May 31, 1995, now U.S. Patent No. 5,792,846, which is a continuation of U.S. application Serial No. 07/868,354, filed April 10, 1992.

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## LOW-VOLTAGE ACTIVATED CALCIUM CHANNEL COMPOSITIONS AND METHODS

### RELATED APPLICATIONS

- Benefit of priority to U.S. application Serial No. 08/984,709, to Williams *et al.*, entitled, "CALCIUM CHANNEL COMPOSITIONS AND METHODS" filed December 3, 1997, and to U.S. application Serial No. 09/188,932, to Williams *et al.*, entitled, "CALCIUM CHANNEL COMPOSITIONS AND METHODS" filed November 10, 1998 is claimed herein.
- 10 This application is related to U.S. application Serial No. 08/450,272, filed May 25, 1995, U.S. application Serial No. 08/450,273, filed May 25, 1995, U.S. application Serial No. 08/450,562, filed May 25, 1995. Each of these applications is a continuation-in-part of U.S. application Serial No. 08/290,012. This
- 15 application is also related to International PCT application No. PCT/US94/09230, filed August 11, 1994, which claims priority to U.S. application Serial Nos. 08/105,536 and 08/149,097.
- This application is also related to U.S. application Serial No. 08/404,354, filed February 15, 1995, now U.S. Patent No. 5,618,720, which is a
- 20 continuation of U.S. application Serial No. 07/914,231, filed July 13, 1992, now U.S. Patent No. 5,407,820, and also U.S. application Serial No. 08/314,083, filed September 28, 1994, now U.S. Patent No. 5,686,241, U.S. application Serial No. 08/435,675, filed May 5, 1995, now U.S. Patent No. 5,710,250, each of which is a divisional of U.S.
- 25 application Serial No. 07/914,231. U.S. application Serial No. 07/914,231 is a continuation of U.S. application Serial No. 07/603,751, filed November 8, 1990, now abandoned, which is the national stage of International PCT Application PCT/US89/01408, filed April 4, 1989,

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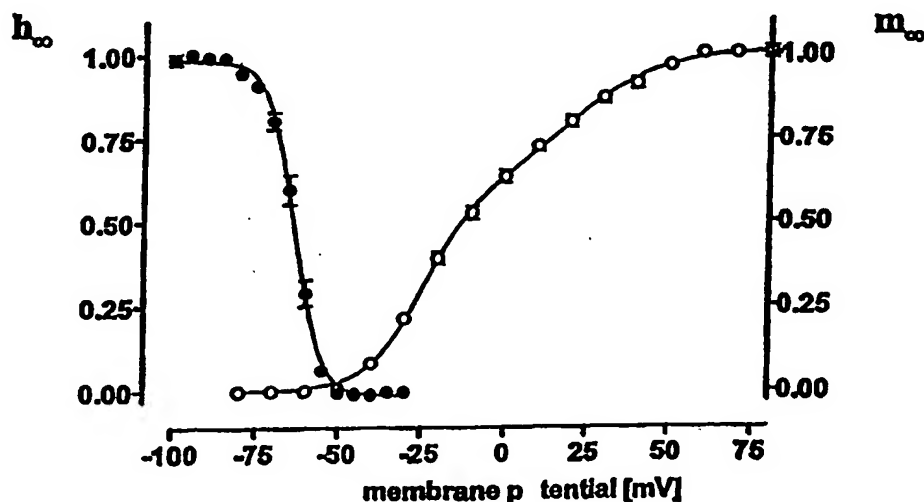


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(54) Title: LOW-VOLTAGE ACTIVATED CALCIUM CHANNEL COMPOSITIONS AND METHODS

## Steady-state activation and inactivation



## (57) Abstract

Isolated nucleic acid encoding low voltage activated calcium channel subunits, including subunits encoded by nucleic acid that arises as splice variants of primary transcripts, is provided. Cells and vectors containing the nucleic acid and methods for identifying compounds that modulate the activity of calcium channels that contain these subunits are also provided.

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understanding the observed different broad ranges of values that have been reported for the  $V_{1/2}$  inactivation of T-type calcium channels (-100 to -50 mV) in different tissues (see, *e.g.*, Huguenard (1996) Annual Rev. Physiol. 58:329-348).

#### 5 F. Summary of Biophysical Properties of Human $\alpha_{1H}$ Containing calcium channels

TABLE 5 summarizes the biophysical properties of calcium channels containing the human  $\alpha_{1H}$  subunits.

TABLE 5

10 Comparison of biophysical parameters of  $\alpha_{1H}$  subunits transiently expressed in HEK293 cells using 15 mM  $Ba^{2+}$  as the charge carrier:

	Parameter	$\alpha_{1H-1}$	$\alpha_{1H-2}$	Statistical significance
15 Current voltage relationship	max current at x [mV]	-10	-20	$p < 0.05$
Isochronic inactivation (20 seconds)	$V_{1/2}$ [mV]	-62.5	-73	$p < 0.05$
	Slope	-3.45	-3.82	no (0.279)
Steady-state activation	$V_{1/2,A}$ [mV]	-23.7	-33.8	$p < 0.05$
	Slope <sub>A</sub>	8.03	5.51	$p < 0.05$
	Fraction <sub>A</sub>	0.617	0.519	no (0.133)
	$V_{1/2,B}$ [mV]	23.1	10.7	$p < 0.05$
	Slope <sub>B</sub>	10.9	11.6	no (0.742)

20  $\alpha_{1H-1}$  corresponds to the wild type form of the subunit;  $\alpha_{1H-2}$  to the splice variant form;

Steady-state activation from Boltzman fit in the form:  $m_{\infty} = \text{Fraction}_A \cdot$

$[1 + \exp(-(V_{\text{test}} - V_{1/2,A})/\text{Slope}_A)]^{-1} + (1 - \text{Fraction}_A) \cdot [1 + \exp(-(V_{\text{test}} -$

25  $V_{1/2,B}/\text{Slope}_B)]^{-1}$ ; Isochronic inactivation (or steady-state inactivation) from Boltzman fit in the form:  $h_{\infty} = [1 + \exp((V_{\text{test}} - V_{1/2})/\text{Slope})]^{-1}$

#### G. Pharmacologic Profile of Human $\alpha_{1H}$ calcium channels

The sensitivity of  $\alpha_{1H}Ca^{2+}$  channels expressed in HEK293 cells to  
 30 several agents known to act on VGCCs (Table below) was tested.  $\alpha_{1H}$ -mediated currents were 16-fold more sensitive to  $Ni^{2+}$  ( $IC_{50} = 6.6 \mu M$ ) than to  $Cd^{2+}$  ( $IC_{50} = 104 \mu M$ ). Currents were also inhibited by the T-type

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channel antagonists amiloride ( $IC_{50} = 167 \mu M$ ) and mibefradil ( $51.0 \pm 10.0\%$  at  $1 \mu M$ ;  $n = 5$ ). In contrast, the T-type channel antagonist ethosuximide produced little inhibition of  $\alpha_{1H}$ -mediated currents ( $7.2 \pm 1.8\%$  inhibition at  $300 \mu M$ ;  $n = 5$ ). The calcium channel inhibitor verapamil, the L-type antagonist nimodipine, and the L-type agonist (-)-Bay K 8644 had little effect on  $\alpha_{1H}$  channels at a concentration of  $1 \mu M$ . A higher concentration ( $10 \mu M$ ) of nimodipine or (-)-Bay K 8644 produced a marked inhibition ( $43.7 \pm 4.1\%$ ,  $n = 4$ , and  $18.1 \pm 9.1\%$ ,  $n = 5$ , respectively). The peptide toxins  $\omega$ -CgTx GVIA and  $\omega$ -CmTx MVIIC at a concentration of  $1 \mu M$  provided little or no inhibition of  $\alpha_{1H}$ -mediated currents.

Pharmacological studies reveal the following rank order of potency for inhibition of  $\alpha_{1H.1}$ -containing channels:  $ni^{2+}$  ( $IC_{50}$ :  $6.6 \mu M$ )  $\approx$  Mibefradil ( $51\%$  at  $1 \mu M$ )  $>$   $Cd^{2+}$  ( $IC_{50}$ :  $104 \mu M$ )  $>$  Amiloride ( $IC_{50}$ :  $167 \mu M$ )  $>>$  Ethosuximide ( $7\%$  at  $300 \mu M$ ). Nimodipine, Verapamil,  $\omega$ -CgTx GVIA and  $\omega$ -CmTx MVIIC had little effect ( $0-17\%$ ) at a concentration of  $1 \mu M$ . These findings demonstrate that  $\alpha_{1H}$ -containing calcium channels have properties corresponding to native LVA, or T-type calcium channels.

Table 6 summarizes the pharmacological profile of human  $\alpha_{1H}$  containing calcium channels expressed in HEK293 cells. With the exception of  $\omega$ -CmTx MVIIC, in all cases the charge carrier was  $15 \text{ mM Ba}^{2+}$ . In the case of  $\omega$ -CmTx MVIIC the charge carrier for was  $2 \text{ mM Ba}^{2+}$  because  $\omega$ -CmTx MVIIC was a more effective inhibitor at lower divalent concentrations. Values for % block are mean  $\pm$  SD( $n$ ).  $IC_{50}$  values were calculated from sigmoidal curve fitting data (Prism, Graphpad Inc.) for data points from 3 to 6 determinations.

**TABLE 6**  
**Pharmacology of  $\alpha_{1H}$   $Ca^{2+}$  Channels Expressed in HEK293 Cells**

	Compound	Concentration	% Inhibition of Control Response or IC <sub>50</sub>
5	Cd <sup>2+</sup>	range	104 $\mu$ M
	Ni <sup>2+</sup>	range	6.6 $\mu$ M
	Amiloride	range	167 $\mu$ M
	Mibefradil	1 $\mu$ M	51.0 $\pm$ 10.0%(5)
	Ethosuximide	300 $\mu$ M	7.2 $\pm$ 1.8%(5)
10	Verapamil	1 $\mu$ M	17.2 $\pm$ 1.3%(3)
	Nimodipine	1 $\mu$ M	3.4 $\pm$ 1.1%(4)
	(-)-BayK-8644	10 $\mu$ M	43.7 $\pm$ 4.1%(4)
		1 $\mu$ M	0.4 $\pm$ 0.8%(3)
15	$\omega$ -CgTx	10 $\mu$ M	18.1 $\pm$ 9.1%(5)
	GVIA	1 $\mu$ M	0%(3)
	$\omega$ -CmTx		
	MV1IC	1 $\mu$ M	8.6 $\pm$ 11.5%(3)

**20 EXAMPLE 4: RECOMBINANT EXPRESSION OF HUMAN NEURONAL CALCIUM CHANNEL SUBUNIT-ENCODING cDNA AND RNA TRANSCRIPTS IN MAMMALIAN CELLS**

The methods and assays described in this example, may be employed using the nucleic encoding an  $\alpha_{1H}$  subunit in place of the  $\alpha_1$  subunits exemplified below. Of particular interest are cells that express the  $\alpha_{1H}$  subunit alone, as homomers, monomers or multimers, or in combination with selected  $\alpha_2$  subunits.

**30 A. Recombinant Expression of the Human Neuronal Calcium Channel  $\alpha_2$  subunit cDNA in DG44 Cells**

**1. Stable transfection of DG44 cells**

DG44 cells (dhfr<sup>-</sup> Chinese hamster ovary cells; see, *e.g.*, Urlaub, G. *et al.* (1986) *Som. Cell Molec. Genet.* 12:555-566) obtained from Lawrence Chasin at Columbia University were stably transfected by CaPO<sub>4</sub> precipitation methods (Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. USA* 76:1373-1376) with pSV2dhfr vector containing the human neuronal calcium channel  $\alpha_2$ -subunit cDNA for polycistronic

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expression/selection in transfected cells. Transfectants were grown on 10% DMEM medium without hypoxanthine or thymidine in order to select cells that had incorporated the expression vector. Twelve transfectant cell lines were established as indicated by their ability to survive on this  
5 medium.

**2. Analysis of  $\alpha_2$  subunit cDNA expression in transfected DG44 cells**

Total RNA was extracted according to the method of Birnboim ((1988) *Nuc. Acids Res.* 16:1487-1497) from four of the DG44 cell lines  
10 that had been stably transfected with pSV2dhfr containing the human neuronal calcium channel  $\alpha_2$  subunit cDNA. RNA ( $\sim 15 \mu\text{g}$  per lane) was separated on a 1% agarose formaldehyde gel, transferred to nitrocellulose and hybridized to the random-primed human neuronal calcium channel  $\alpha_2$  cDNA (hybridization: 50% formamide, 5 x SSPE, 5 x Denhardt's, 42° C.;  
15 wash :0.2 x SSPE, 0.1% SDS, 65° C.). Northern blot analysis of total RNA from four of the DG44 cell lines that had been stably transfected with pSV2dhfr containing the human neuronal calcium channel  $\alpha_2$  subunit cDNA revealed that one of the four cell lines contained hybridizing mRNA the size expected for the transcript of the  $\alpha_2$  subunit cDNA (5000 nt  
20 based on the size of the cDNA) when grown in the presence of 10 mM sodium butyrate for two days. Butyrate nonspecifically induces transcription and is often used for inducing the SV40 early promoter (Gorman, C. and Howard, B. (1983) *Nucleic Acids Res.* 11:1631). This cell line, 44 $\alpha_2$ -9, also produced mRNA species smaller (several species)  
25 and larger (6800 nt) than the size expected for the transcript of the  $\alpha_2$  cDNA (5000 nt) that hybridized to the  $\alpha_2$  cDNA-based probe. The 5000- and 6800-nt transcripts produced by this transfectant should contain the entire  $\alpha_2$  subunit coding sequence and therefore should yield a full-length  $\alpha_2$  subunit protein. A weakly hybridizing 8000-nucleotide transcript was

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present in untransfected and transfected DG44 cells. Apparently, DG44 cells transcribe a calcium channel  $\alpha_2$  subunit or similar gene at low levels. The level of expression of this endogenous  $\alpha_2$  subunit transcript did not appear to be affected by exposing the cells to butyrate before isolation of

5 RNA for northern analysis.

Total protein was extracted from three of the DG44 cell lines that had been stably transfected with pSV2dhfr containing the human neuronal calcium channel  $\alpha_2$  subunit cDNA. Approximately  $10^7$  cells were sonicated in 300  $\mu$ l of a solution containing 50 mM HEPES, 1 mM EDTA,

10 1 mM PMSF. An equal volume of 2x loading dye (Laemmli, U.K. (1970). *Nature* 227:680) was added to the samples and the protein was subjected to electrophoresis on an 8% polyacrylamide gel and then electrotransferred to nitrocellulose. The nitrocellulose was incubated with polyclonal guinea pig antisera (1:200 dilution) directed against the rabbit

15 skeletal muscle calcium channel  $\alpha_2$  subunit (obtained from K. Campbell, University of Iowa) followed by incubation with [ $^{125}$ I]-protein A. The blot was exposed to X-ray film at  $-70^\circ$  C. Reduced samples of protein from the transfected cells as well as from untransfected DG44 cells contained immunoreactive protein of the size expected for the  $\alpha_2$  subunit of the

20 human neuronal calcium channel (130-150 kDa). The level of this immunoreactive protein was higher in 44 $\alpha_2$ -9 cells that had been grown in the presence of 10 mM sodium butyrate than in 44 $\alpha_2$ -9 cells that were grown in the absence of sodium butyrate. These data correlate well with those obtained in northern analyses of total RNA from 44 $\alpha_2$ -9 and

25 untransfected DG44 cells. Cell line 44 $\alpha_2$ -9 also produced a 110 kD immunoreactive protein that may be either a product of proteolytic degradation of the full-length  $\alpha_2$  subunit or a product of translation of one of the shorter (<5000 nt) mRNA produced in this cell line that hybridized to the  $\alpha_2$  subunit cDNA probe.

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**B. Expression of DNA encoding human neuronal calcium channel  $\alpha_1$ ,  $\alpha_2$  and  $\beta_1$  subunits in HEK cells**

Human embryonic kidney cells (HEK 293 cells) were transiently and stably transfected with human neuronal DNA encoding calcium channel subunits. Individual transfectants were analyzed electrophysiologically for the presence of voltage-activated barium currents and functional recombinant voltage-dependent calcium channels were analyzed.

**1. Transfection of HEK 293 cells**

Separate expression vectors containing DNA encoding human neuronal calcium channel  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunits, plasmids pVDCCIII(A), pHBCaCH $\alpha_2$ A, and pHBCaCH $\beta_{1a}$ RBS(A), respectively, were constructed as described in International PCT application No. PCT/US94/09230, see, also allowed U.S. application Serial No. 08/149,097. These three vectors were used to transiently co-transfect HEK 293 cells. For stable transfection of HEK 293 cells, vector pHBCaCH $\beta_{1b}$ RBS(A) was used in place of pHBCaCH $\beta_{1a}$ RBS(A) to introduce the DNA encoding the  $\beta_1$  subunit into the cells along with pVDCCIII(A) and pHBCaCH $\alpha_2$ A.

**a. Transient transfection**

Expression vectors pVDCCIII(A), pHBCaCH $\alpha_2$ A and pHBCaCH $\beta_{1a}$ RBS(A) were used in two sets of transient transfections of HEK 293 cells (ATCC Accession No. CRL1573). In one transfection procedure, HEK 293 cells were transiently cotransfected with the  $\alpha_1$  subunit cDNA expression plasmid, the  $\alpha_2$  subunit cDNA expression plasmid, the  $\beta_1$  subunit cDNA expression plasmid and plasmid pCMV $\beta$ gal (Clontech Laboratories, Palo Alto, CA). Plasmid pCMV $\beta$ gal contains the *lacZ* gene (encoding *E. coli*  $\beta$ -galactosidase) fused to the cytomegalovirus (CMV) promoter and was included in this transfection as a marker gene for monitoring the efficiency of transfection. In the other transfection procedure, HEK 293 cells were transiently co-transfected with the  $\alpha_1$

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subunit cDNA expression plasmid pVDCCIII(A) and pCMV $\beta$ gal. In both transfections,  $2-4 \times 10^6$  HEK 293 cells in a 10-cm tissue culture plate were transiently co-transfected with 5  $\mu$ g of each of the plasmids included in the experiment according to standard  $\text{CaPO}_4$  precipitation transfection procedures (Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. USA* 76:1373-1376). The transfectants were analyzed for  $\beta$ -galactosidase expression by direct staining of the product of a reaction involving  $\beta$ -galactosidase and the X-gal substrate (Jones, J.R. (1986) *EMBO* 5:3133-3142) and by measurement of  $\beta$ -galactosidase activity (Miller, J.H. (1972) Experiments in Molecular Genetics, pp. 352-355, Cold Spring Harbor Press). To evaluate subunit cDNA expression in these transfectants, the cells were analyzed for subunit transcript production (northern analysis), subunit protein production (immunoblot analysis of cell lysates) and functional calcium channel expression (electrophysiological analysis).

15                                    **b. Stable transfection**

HEK 293 cells were transfected using the calcium phosphate transfection procedure (*Current Protocols in Molecular Biology*, Vol. 1, Wiley Inter-Science, Supplement 14, Unit 9.1.1-9.1.9 (1990)). Ten-cm plates, each containing one-to-two million HEK 293 cells, were transfected with 1 ml of DNA/calcium phosphate precipitate containing 5  $\mu$ g pVDCCIII(A), 5  $\mu$ g pHBCaCH $\alpha_2$ A, 5  $\mu$ g pHBCaCH $\beta_{1b}$ RBS(A), 5  $\mu$ g pCMVBgal and 1  $\mu$ g pSV2neo (as a selectable marker). After 10-20 days of growth in media containing 500  $\mu$ g G418, colonies had formed and were isolated using cloning cylinders.

25                                    **2. Analysis of HEK 293 cells transiently transfected with DNA encoding human neuronal calcium channel subunits**

**a. Analysis of  $\beta$ -galactosidase expression**

Transient transfectants were assayed for  $\beta$ -galactosidase expression by  $\beta$ -galactosidase activity assays (Miller, J.H., (1972)

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Experiments in Molecular Genetics, pp. 352-355, Cold Spring Harbor Press) of cell lysates (prepared as described in International PCT application No. PCT/US94/09230, see, also allowed U.S. application Serial No. 08/149,097) and staining of fixed cells (Jones, J.R. (1986) *EMBO* 5:3133-3142). The results of these assays indicated that approximately 30% of the HEK 293 cells had been transfected.

**b. Northern analysis**

PolyA + RNA was isolated using the Invitrogen Fast Trak Kit (Invitrogen, San Diego, CA) from HEK 293 cells transiently transfected with DNA encoding each of the  $\alpha_1$ ,  $\alpha_2$  and  $\beta_1$  subunits and the *lacZ* gene or the  $\alpha_1$  subunit and the *lacZ* gene. The RNA was subjected to electrophoresis on an agarose gel and transferred to nitrocellulose. The nitrocellulose was then hybridized with one or more of the following radiolabeled probes: the *lacZ* gene, human neuronal calcium channel  $\alpha_{1D}$  subunit-encoding cDNA, human neuronal calcium channel  $\alpha_2$  subunit-encoding cDNA or human neuronal calcium channel  $\beta_1$  subunit-encoding cDNA. Two transcripts that hybridized with the  $\alpha_1$  subunit-encoding cDNA were detected in HEK 293 cells transfected with the DNA encoding the  $\alpha_1$ ,  $\alpha_2$ , and  $\beta_1$  subunits and the *lacZ* gene as well as in HEK 293 cells transfected with the  $\alpha_1$  subunit cDNA and the *lacZ* gene. One mRNA species was the size expected for the transcript of the  $\alpha_1$  subunit cDNA (8000 nucleotides). The second RNA species was smaller (4000 nucleotides) than the size expected for this transcript. RNA of the size expected for the transcript of the *lacZ* gene was detected in cells transfected with the  $\alpha_1$ ,  $\alpha_2$  and  $\beta_1$  subunit-encoding cDNA and the *lacZ* gene and in cells transfected with the  $\alpha_1$  subunit cDNA and the *lacZ* gene by hybridization to the *lacZ* gene sequence.

RNA from cells transfected with the  $\alpha_1$ ,  $\alpha_2$  and  $\beta_1$  subunit-encoding cDNA and the *lacZ* gene was also hybridized with the  $\alpha_2$  and  $\beta_1$  subunit



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cDNA probes. Two mRNA species hybridized to the  $\alpha_2$  subunit cDNA probe. One species was the size expected for the transcript of the  $\alpha_2$  subunit cDNA (4000 nucleotides). The other species was larger (6000 nucleotides) than the expected size of this transcript. Multiple RNA  
5 species in the cells co-transfected with  $\alpha_1$ ,  $\alpha_2$  and  $\beta_1$  subunit-encoding cDNA and the *lacZ* gene hybridized to the  $\beta_1$  subunit cDNA probe. Multiple  $\beta$  subunit transcripts of varying sizes were produced since the  $\beta$  subunit cDNA expression vector contains two potential polyA<sup>+</sup> addition sites.

10

### c. Electrophysiological analysis

Individual transiently transfected HEK 293 cells were assayed for the presence of voltage-dependent barium currents using the whole-cell variant of the patch clamp technique (Hamill et al. (1981). *Pflugers Arch.* 391:85-100). HEK 293 cells transiently transfected with pCMV $\beta$ gal only  
15 were assayed for barium currents as a negative control in these experiments. The cells were placed in a bathing solution that contained barium ions to serve as the current carrier. Choline chloride, instead of NaCl or KCl, was used as the major salt component of the bath solution to eliminate currents through sodium and potassium channels. The  
20 bathing solution contained 1 mM MgCl<sub>2</sub> and was buffered at pH 7.3 with 10 mM HEPES (pH adjusted with sodium or tetraethylammonium hydroxide). Patch pipettes were filled with a solution containing 135 mM CsCl, 1 mM MgCl<sub>2</sub>, 10 mM glucose, 10 mM EGTA, 4 mM ATP and 10 mM HEPES (pH adjusted to 7.3 with tetraethylammonium hydroxide).  
25 Cesium and tetraethylammonium ions block most types of potassium channels. Pipettes were coated with Sylgard (Dow-Corning, Midland, MI) and had resistances of 1-4 megohm. Currents were measured through a 500 megohm headstage resistor with the Axopatch IC (Axon Instruments, Foster City, CA) amplifier, interfaced with a Labmaster (Scientific

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Solutions, Solon, OH) data acquisition board in an IBM-compatible PC. PClamp (Axon Instruments) was used to generate voltage commands and acquire data. Data were analyzed with pClamp or Quattro Professional (Borland International, Scotts Valley, CA) programs.

- 5 To apply drugs, "puffer" pipettes positioned within several micrometers of the cell under study were used to apply solutions by pressure application. The drugs used for pharmacological characterization were dissolved in a solution identical to the bathing solution. Samples of a 10 mM stock solution of Bay K 8644 (RBI, Natick, MA), which was  
10 prepared in DMSO, were diluted to a final concentration of 1  $\mu$ M in 15 mM  $\text{Ba}^{2+}$ -containing bath solution before they were applied.

- Twenty-one negative control HEK 293 cells (transiently transfected with the *lacZ* gene expression vector pCMV $\beta$ gal only) were analyzed by the whole-cell variant of the patch clamp method for recording currents.  
15 Only one cell displayed a discernable inward barium current; this current was not affected by the presence of 1  $\mu$ M Bay K 8644. In addition, application of Bay K 8644 to four cells that did not display  $\text{Ba}^{2+}$  currents did not result in the appearance of any currents.

- Two days after transient transfection of HEK 293 cells with  $\alpha_1$ ,  $\alpha_2$   
20 and  $\beta_1$  subunit-encoding cDNA and the *lacZ* gene, individual transfectants were assayed for voltage-dependent barium currents. The currents in nine transfectants were recorded. Because the efficiency of transfection of one cell can vary from the efficiency of transfection of another cell, the degree of expression of heterologous proteins in individual transfectants  
25 varies and some cells do not incorporate or express the foreign DNA. Inward barium currents were detected in two of these nine transfectants. In these assays, the holding potential of the membrane was -90 mV. The membrane was depolarized in a series of voltage steps to different test potentials and the current in the presence and absence of 1  $\mu$ M Bay K

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8644 was recorded. The inward barium current was significantly enhanced in magnitude by the addition of Bay K 8644. The largest inward barium current ( $\sim 160$  pA) was recorded when the membrane was depolarized to 0 mV in the presence of  $1 \mu\text{M}$  Bay K 8644. A comparison  
5 of the I-V curves, generated by plotting the largest current recorded after each depolarization versus the depolarization voltage, corresponding to recordings conducted in the absence and presence of Bay K 8644 illustrated the enhancement of the voltage-activated current in the presence of Bay K 8644.

10 Pronounced tail currents were detected in the tracings of currents generated in the presence of Bay K 8644 in HEK 293 cells transfected with  $\alpha_1$ ,  $\alpha_2$  and  $\beta_1$  subunit-encoding cDNA and the *lacZ* gene, indicating that the recombinant calcium channels responsible for the voltage-activated barium currents recorded in this transfected appear to be DHP-  
15 sensitive.

The second of the two transfected cells that displayed inward barium currents expressed a  $\sim 50$  pA current when the membrane was depolarized from -90 mV. This current was nearly completely blocked by  $200 \mu\text{M}$  cadmium, an established calcium channel blocker.

20 Ten cells that were transiently transfected with the DNA encoding the  $\alpha_1$  subunit and the *lacZ* gene were analyzed by whole-cell patch clamp methods two days after transfection. One of these cells displayed a 30 pA inward barium current. This current amplified 2-fold in the presence of  $1 \mu\text{M}$  Bay K 8644. Furthermore, small tail currents were detected in  
25 the presence of Bay K 8644. These data indicate that expression of the human neuronal calcium channel  $\alpha_{1D}$  subunit-encoding cDNA in HEK 293 yields a functional DHP-sensitive calcium channel.

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### 3. Analysis of HEK 293 cells stably transfected with DNA encoding human neuronal calcium channel subunits

Individual stably transfected HEK 293 cells were assayed electrophysiologically for the presence of voltage-dependent barium currents as described for electrophysiological analysis of transiently transfected HEK 293 cells (International PCT application No. PCT/US94/09230, see, also allowed U.S. application Serial No. 08/149,097). In an effort to maximize calcium channel activity via cyclic-AMP-dependent kinase-mediated phosphorylation (Pelzer, et al. (1990) *Rev. Physiol. Biochem. Pharmacol.* 114:107-207), cAMP (Na salt, 250  $\mu$ M) was added to the pipet solution and forskolin (10  $\mu$ M) was added to the bath solution in some of the recordings. Qualitatively similar results were obtained whether these compounds were present or not.

Barium currents were recorded from stably transfected cells in the absence and presence of Bay K 8644 (1  $\mu$ M). When the cell was depolarized to -10 mV from a holding potential of -90 mV in the absence of Bay K 8644, a current of approximately 35pA with a rapidly deactivating tail current was recorded. During application of Bay K 8644, an identical depolarizing protocol elicited a current of approximately 75 pA, accompanied by an augmented and prolonged tail current. The peak magnitude of currents recorded from this same cell as a function of a series of depolarizing voltages were assessed. The responses in the presence of Bay K 8644 not only increased, but the entire current-voltage relation shifted about -10 mV. Thus, three typical hallmarks of Bay K 8644 action, namely increased current magnitude, prolonged tail currents, and negatively shifted activation voltage, were observed, clearly indicating the expression of a DHP-sensitive calcium channel in these stably transfected cells. No such effects of Bay K 8644 were observed in untransfected HEK 293 cells, either with or without cAMP or forskolin.

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**C. Use of pCMV-based vectors and pcDNA1-based vectors for expression of DNA encoding human neuronal calcium channel subunits**

**1. Preparation of constructs**

- 5 Additional expression vectors were constructed using pCMV. The full-length  $\alpha_{1D}$  cDNA from pVDCCIII(A) (see International PCT application No. PCT/US94/09230, see, also allowed U.S. application Serial No. 08/149,097), the full-length  $\alpha_2$  cDNA, contained on a 3600 bp *EcoRI* fragment from HBCaCH $\alpha_2$  (International PCT application No. PCT/US94/09230, see, also allowed U.S. application Serial No. 08/149,097) and a full-length  $\beta_1$  subunit cDNA from pHBCaCH $\beta_1$ RBS(A) (see International PCT application No. PCT/US94/09230, see, also allowed U.S. application Serial No. 08/149,097) were separately subcloned into plasmid pCMV $\beta$ gal. Plasmid pCMV $\beta$ gal was digested with
- 15 *NotI* to remove the *lacZ* gene. The remaining vector portion of the plasmid, referred to as pCMV, was blunt-ended at the *NotI* sites. The full-length  $\alpha_2$ -encoding DNA and  $\beta_1$ -encoding DNA, contained on separate *EcoRI* fragments, were isolated, blunt-ended and separately ligated to the blunt-ended vector fragment of pCMV locating the DNA between the
- 20 CMV promoter and SV40 polyadenylation sites in pCMV. To ligate the  $\alpha_{1D}$ -encoding cDNA with pCMV, the restriction sites in the polylinkers immediately 5' of the CMV promoter and immediately 3' of the SV40 polyadenylation site were removed from pCMV. A polylinker was added at the *NotI* site. The polylinker had the following sequence of restriction
- 25 enzyme recognition sites:

	GGCCGC		<i>EcoRI</i>		<i>SalI</i>		<i>PstI</i>		<i>EcoRV</i>		<i>HindIII</i>		<i>XbaI</i>		GT
	CG		site		site		site		site		site		site		CACCGG
30	<i>NotI</i>														

Destroys *NotI*

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The  $\alpha_{1D}$ -encoding DNA, isolated as a *Bam*HI/*Xho*I fragment from pVDCCIII(A), was then ligated to *Xba*II/*Sa*I-digested pCMV to place it between the CMV promoter and SV40 polyadenylation site.

Plasmid pCMV contains the CMV promoter as does pcDNA1, but  
5 differs from pcDNA1 in the location of splice donor/splice acceptor sites relative to the inserted subunit-encoding DNA. After inserting the subunit-encoding DNA into pCMV, the splice donor/splice acceptor sites are located 3' of the CMV promoter and 5' of the subunit-encoding DNA start codon. After inserting the subunit-encoding DNA into pcDNA1, the  
10 splice donor/splice acceptor sites are located 3' of the subunit cDNA stop codon.

## 2. Transfection of HEK 293 cells

HEK 293 cells were transiently co-transfected with the  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunit-encoding DNA in pCMV or with the  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta$  subunit-  
15 encoding DNA in pcDNA1 (vectors pVDCCIII(A), pHBCaCH $\alpha_2$ A and pHBCaCH $\beta_{1b}$ RBS(A), respectively (see, International PCT application No. PCT/US94/09230, see, also allowed U.S. application Serial No. 08/149,097). Plasmid pCMV $\beta$ gal was included in each transfection as a measure of transfection efficiency. The results of  $\beta$ -galactosidase assays  
20 of the transfectants (International PCT application No. PCT/US94/09230, see, also allowed U.S. application Serial No. 08/149,097), indicated that HEK 293 cells were transfected equally efficiently with pCMV- and pcDNA1-based plasmids. The pcDNA1-based plasmids, however, are presently preferred for expression of calcium channel receptors.

## 25 D. Expression in *Xenopus laevis* oocytes of RNA encoding human neuronal calcium channel subunits

Various combinations of the transcripts of DNA encoding the human neuronal  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunits prepared *in vitro* were injected

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into *Xenopus laevis* oocytes. Those injected with combinations that included  $\alpha_{1D}$  exhibited voltage-activated barium currents.

### 1. Preparation of transcripts

Transcripts encoding the human neuronal calcium channel  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunits were synthesized according to the instructions of the mCAP mRNA CAPPING KIT (Strategene, La Jolla, CA catalog #200350). As described in International PCT application No. PCT/US94/09230, see, also allowed U.S. application Serial No. 08/149,097, plasmids pVDCC III.RBS(A), containing pcDNA1 and the  $\alpha_{1D}$  cDNA that begins with a ribosome binding site and the eighth ATG codon of the coding sequence plasmid pHBCaCH $\alpha_1$ A containing pcDNA1 and an  $\alpha_2$  subunit cDNA, and plasmid pHBCaCH $\beta_{1b}$ RBS(A) containing pcDNA1 and the  $\beta_1$  DNA lacking intron sequence and containing a ribosome binding site were linearized by restriction digestion. The  $\alpha_{1D}$  cDNA- and  $\alpha_2$  subunit-encoding plasmids were digested with *Xho*I, and the  $\beta_1$  subunit- encoding plasmid was digested with *Eco*RV. The DNA insert was transcribed with T7 RNA polymerase.

### 2. Injection of oocytes

*Xenopus laevis* oocytes were isolated and defolliculated by collagenase treatment and maintained in 100 mM NaCl, 2 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 5 mM HEPES, pH 7.6, 20  $\mu\text{g/ml}$  ampicillin and 25  $\mu\text{g/ml}$  streptomycin at 19-25°C for 2 to 5 days after injection and prior to recording. For each transcript that was injected into the oocyte, 6 ng of the specific mRNA was injected per cell in a total volume of 50 nl.

### 3. Intracellular voltage recordings

Injected oocytes were examined for voltage-dependent barium currents using two-electrode voltage clamp methods (Dascal, N. (1987) *CRC Crit. Rev. Biochem.* 22:317). The pClamp (Axon Instruments) software package was used in conjunction with a Labmaster 125 kHz

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data acquisition interface to generate voltage commands and to acquire and analyze data. Quattro Professional was also used in this analysis. Current signals were digitized at 1-5 kHz, and filtered appropriately. The bath solution contained of the following: 40 mM BaCl<sub>2</sub>, 36 mM

5 tetraethylammonium chloride (TEA-Cl), 2 mM KCl, 5 mM 4-aminopyridine, 0.15 mM niflumic acid, 5 mM HEPES, pH 7.6.

a. **Electrophysiological analysis of oocytes injected with transcripts encoding the human neuronal calcium channel  $\alpha_1$ ,  $\alpha_2$  and  $\beta_1$ -subunits**

10 Uninjected oocytes were examined by two-electrode voltage clamp methods and a very small (25 nA) endogenous inward Ba<sup>2+</sup> current was detected in only one of seven analyzed cells.

Oocytes coinjected with  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunit transcripts expressed sustained inward barium currents upon depolarization of the  
15 membrane from a holding potential of -90 mV or -50 mV ( $154 \pm 129$  nA,  $n = 21$ ). These currents typically showed little inactivation when test pulses ranging from 140 to 700 msec. were administered. Depolarization to a series of voltages revealed currents that first appeared at approximately -30 mV and peaked at approximately 0 mV.

20 Application of the DHP Bay K 8644 increased the magnitude of the currents, prolonged the tail currents present upon repolarization of the cell and induced a hyperpolarizing shift in current activation. Bay K 8644 was prepared fresh from a stock solution in DMSO and introduced as a 10x concentrate directly into the 60  $\mu$ l bath while the perfusion pump was  
25 turned off. The DMSO concentration of the final diluted drug solutions in contact with the cell never exceeded 0.1%. Control experiments showed that 0.1% DMSO had no effect on membrane currents.

Application of the DHP antagonist nifedipine (stock solution prepared in DMSO and applied to the cell as described for application of  
30 Bay K 8644) blocked a substantial fraction ( $91 \pm 6\%$ ,  $n = 7$ ) of the



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inward barium current in oocytes coinjected with transcripts of the  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunits. A residual inactivating component of the inward barium current typically remained after nifedipine application. The inward barium current was blocked completely by 50  $\mu\text{M}$   $\text{Cd}^{2+}$ , but only approximately  
5 15% by 100  $\mu\text{M}$   $\text{Ni}^{2+}$ .

The effect of  $\omega$ -CgTX-GVIA on the inward barium currents in oocytes co-injected with transcripts of the  $\alpha_{1D}$ ,  $\alpha_2$ , and  $\beta_1$  subunits was investigated.  $\omega$ -CgTX-GVIA (Bachem, Inc., Torrance CA) was prepared in the 15 mM  $\text{BaCl}_2$  bath solution plus 0.1% cytochrome C (Sigma) to serve  
10 as a carrier protein. Control experiments showed that cytochrome C had no effect on currents. A series of voltage pulses from a -90 mV holding potential to 0 mV were recorded at 20 msec. intervals. To reduce the inhibition of  $\omega$ CgTX binding by divalent cations, recordings were made in 15 mM  $\text{BaCl}_2$ , 73.5 mM tetraethylammonium chloride, and the remaining  
15 ingredients identical to the 40 mM  $\text{Ba}^{2+}$  recording solution. Bay K 8644 was applied to the cell prior to addition to  $\omega$ CgTX in order to determine the effect of  $\omega$ CgTX on the DHP-sensitive current component that was distinguished by the prolonged tail currents. The inward barium current was blocked weakly ( $54 \pm 29\%$ ,  $n=7$ ) and reversibly by relatively high  
20 concentrations (10-15  $\mu\text{M}$ ) of  $\omega$ CgTX. The test currents and the accompanying tail currents were blocked progressively within two to three minutes after application of  $\omega$ CgTX, but both recovered partially as the  $\omega$ CgTX was flushed from the bath.

25                   b.    **Analysis of oocytes injected with transcripts encoding the human neuronal calcium channel  $\alpha_{1D}$  or transcripts encoding an  $\alpha_{1D}$  and other subunits**

The contribution of the  $\alpha_2$  and  $\beta_1$  subunits to the inward barium current in oocytes injected with transcripts encoding the  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$   
30 subunits was assessed by expression of the  $\alpha_{1D}$  subunit alone or in

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combination with either the  $\beta_1$  subunit or the  $\alpha_2$  subunit. In oocytes injected with only the transcript of a  $\alpha_{1D}$  cDNA, no  $Ba^{2+}$  currents were detected ( $n=3$ ). In oocytes injected with transcripts of  $\alpha_{1D}$  and  $\beta_1$  encoding DNA, small ( $108 \pm 39$  nA)  $Ba^{2+}$  currents were detected upon depolarization of the membrane from a holding potential of -90 mV that resembled the currents observed in cells injected with transcripts of  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  encoding DNA, although the magnitude of the current was less. In two of the four oocytes injected with transcripts of the  $\alpha_{1D}$ -encoding and  $\beta_1$ -encoding DNA, the  $Ba^{2+}$  currents exhibited a sensitivity to Bay K 8644 that was similar to the Bay K 8644 sensitivity of  $Ba^{2+}$  currents expressed in oocytes injected with transcripts encoding the  $\alpha_{1D}$ ,  $\alpha_1$ ,  $\alpha_2$  and  $\beta_1$  subunits.

Three of five oocytes injected with transcripts encoding the  $\alpha_{1D}$  and  $\alpha_2$  subunits exhibited very small  $Ba^{2+}$  currents (15-30 nA) upon depolarization of the membrane from a holding potential of -90 mV. These barium currents showed little or no response to Bay K 8644.

**c. Analysis of oocytes injected with transcripts encoding the human neuronal calcium channel  $\alpha_2$  and/or  $\beta_1$  subunit**

To evaluate the contribution of the  $\alpha_{1D}$   $\alpha_1$ -subunit to the inward barium currents detected in oocytes co-injected with transcripts encoding the  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunits, oocytes injected with transcripts encoding the human neuronal calcium channel  $\alpha_2$  and/or  $\beta_1$  subunits were assayed for barium currents. Oocytes injected with transcripts encoding the  $\alpha_2$  subunit displayed no detectable inward barium currents ( $n=5$ ). Oocytes injected with transcripts encoding a  $\beta_1$  subunit displayed measurable ( $54 \pm 23$  nA,  $n=5$ ) inward barium currents upon depolarization and oocytes injected with transcripts encoding the  $\alpha_2$  and  $\beta_1$  subunits displayed inward barium currents that were approximately 50% larger ( $80 \pm 61$  nA,

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n = 18) than those detected in oocytes injected with transcripts of the  $\beta_1$ -encoding DNA only.

The inward barium currents in oocytes injected with transcripts encoding the  $\beta_1$  subunit or  $\alpha_2$  and  $\beta_1$  subunits typically were first observed when the membrane was depolarized to -30 mV from a holding potential of -90 mV and peaked when the membrane was depolarized to 10 to 20 mV. Macroscopically, the currents in oocytes injected with transcripts encoding the  $\alpha_2$  and  $\beta_1$  subunits or with transcripts encoding the  $\beta_1$  subunit were indistinguishable. In contrast to the currents in oocytes co-injected with transcripts of  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunit encoding DNA, these currents showed a significant inactivation during the test pulse and a strong sensitivity to the holding potential. The inward barium currents in oocytes co-injected with transcripts encoding the  $\alpha_2$  and  $\beta_1$  subunits usually inactivated to 10-60% of the peak magnitude during a 140-msec pulse and were significantly more sensitive to holding potential than those in oocytes co-injected with transcripts encoding the  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunits. Changing the holding potential of the membranes of oocytes co-injected with transcripts encoding the  $\alpha_2$  and  $\beta_1$  subunits from -90 to -50 mV resulted in an approximately 81% (n = 11) reduction in the magnitude of the inward barium current of these cells. In contrast, the inward barium current measured in oocytes co-injected with transcripts encoding the  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunits were reduced approximately 24% (n = 11) when the holding potential was changed from -90 to -50 mV.

The inward barium currents detected in oocytes injected with transcripts encoding the  $\alpha_2$  and  $\beta_1$  subunits were pharmacologically distinct from those observed in oocytes co-injected with transcripts encoding the  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunits. Oocytes injected with transcripts encoding the  $\alpha_2$  and  $\beta_1$  subunits displayed inward barium currents that were insensitive to Bay K 8644 (n = 11). Nifedipine sensitivity was

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difficult to measure because of the holding potential sensitivity of nifedipine and the current observed in oocytes injected with transcripts encoding the  $\alpha_2$  and  $\beta_1$  subunits. Nevertheless, two oocytes that were co-injected with transcripts encoding the  $\alpha_2$  and  $\beta_1$  subunits displayed measurable (25 to 45 nA) inward barium currents that were insensitive to nifedipine (5 to 10  $\mu$ M), when depolarized from a holding potential of -50 mV. The inward barium currents in oocytes injected with transcripts encoding the  $\alpha_2$  and  $\beta_1$  subunits showed the same sensitivity to heavy metals as the currents detected in oocytes injected with transcripts encoding the  $\alpha_{1D}$ ,  $\alpha_2$  and  $\beta_1$  subunits.

The inward barium current detected in oocytes injected with transcripts encoding the human neuronal  $\alpha_2$  and  $\beta_1$  subunits has pharmacological and biophysical properties that resemble calcium currents in uninjected *Xenopus* oocytes. Because the amino acids of this human neuronal calcium channel  $\beta_1$  subunit lack hydrophobic segments capable of forming transmembrane domains. It is unlikely that recombinant  $\beta_1$  subunits alone form an ion channel, but rather that an endogenous  $\alpha_1$  subunit exists in oocytes and that the activity mediated by such an  $\alpha_1$  subunit is enhanced by expression of a human neuronal  $\beta_1$  subunit.

20

While the subject matter of the invention has been described with some specificity, modifications apparent to those with ordinary skill in the art may be made without departing from the scope of the invention. Since such modifications will be apparent to those of skill in the art, it is intended that this invention be limited only by the scope of the appended claims.

25

**WHAT IS CLAIMED IS:**

1. An isolated nucleic acid fragment that encodes a low-voltage activated subunit of an animal calcium channel.
2. The nucleic acid of claim 1, wherein the subunit is an  $\alpha_{1H}$ -  
5 subunit.
3. The nucleic acid of claim 2, wherein the calcium channel is a mammalian calcium channel.
4. The isolated nucleic acid fragment of claim 2, comprising a sequence of nucleotides that encodes the subunit, wherein the sequence  
10 of nucleotides encoding the subunit is selected from among:
  - (a) a sequence of nucleotides that encodes a calcium channel subunit and comprises the coding portion of the sequence of nucleotides set forth in any of SEQ ID Nos. 12-16;
  - (b) a sequence of nucleotides that encodes an  $\alpha_{1H}$ -subunit and  
15 hybridizes under conditions of high stringency to DNA that is complementary to an mRNA transcript present in a mammalian cell that encodes an  $\alpha_{1H}$ -subunit;
  - (c) a sequence of nucleotides that encodes the subunit that comprises a sequence of amino acids encoded by any of  
20 SEQ ID Nos. 12-16; and
  - (d) a sequence of nucleotides that is degenerate with any of (a), (b) or (c).
5. The molecule of claim 2, wherein the subunit is an  $\alpha_{1H-1}$  subunit or an  $\alpha_{1H-2}$  subunit.
- 25 6. A eukaryotic cell, comprising heterologous nucleic acid that encodes an  $\alpha_1$ -subunit, wherein the  $\alpha_1$ -subunit is encoded by the nucleic acid of any of claims 1-5.

7        The cell of claim 6, further comprising heterologous nucleic acid that encodes a  $\alpha_2\delta$ -subunit of a calcium channel.

8.       The eukaryotic cell of claim 6 or claim 7 that has a functional heterologous calcium channel that contains at least one subunit encoded  
5       by the heterologous nucleic acid.

9.       The eukaryotic cell of any of claims 6-8 selected from the group consisting of HEK 293 cells, Chinese hamster ovary cells, African green monkey cells, and mouse L cells.

10.      A eukaryotic cell with a functional, heterologous calcium  
10      channel, produced by a process comprising:  
          introducing into the cell heterologous nucleic acid that encodes at least one subunit of a calcium channel, wherein the subunit is encoded by the nucleic acid of any of claims 1-5.

11.      The eukaryotic cell of claim 10 that is an amphibian oöcyte.

15       12.      The eukaryotic cell of claim 8 or claim 10, wherein the heterologous calcium channel comprises a plurality of  $\alpha_{1H}$ -subunits.

13       The eukaryotic cell of claim 12, wherein the  $\alpha_{1H}$ -subunits comprise a homomer.

14.      The eukaryotic cell of any of claims 10-13, further  
20      comprising an  $\alpha_2\delta$ -subunit of a calcium channel.

15.      The eukaryotic cell of claim 10, wherein the heterologous nucleic acid encodes a T-type calcium channel.

16.      The eukaryotic cell of claim 8 with a functional, heterologous calcium channel, produced by a process comprising:  
25      introducing into the cell RNA that encodes an  $\alpha_{1H}$  subunit of a calcium channel and optionally introducing into the cell nucleic acid that encodes a  $\beta$ ,  $\alpha_2\delta$  and/or  $\gamma$ -subunit of a calcium channel, wherein:

the heterologous calcium channel contains at least one subunit encoded by the heterologous nucleic acid; and

the only heterologous ion channels are calcium channels.

17. The eukaryotic cell of claim 8 with a functional, heterologous calcium channel, produced by a process comprising:

introducing into the cell DNA that encodes an  $\alpha_{1H}$  subunit of a calcium channel and optionally introducing into the cell nucleic acid that encodes a  $\beta$ ,  $\alpha_2\delta$  and/or  $\gamma$ -subunit of a calcium channel, wherein:

- the heterologous calcium channel contains at least one subunit encoded by the heterologous nucleic acid.

18. The eukaryotic cell of claim 17 selected from the group consisting of HEK 293 cells, Chinese hamster ovary cells, African green monkey cells, mouse L cells and amphibian oocytes.

19. The eukaryotic cell of claim 16 selected from the group consisting of amphibian oocytes.

20. The eukaryotic cell of any of claims 6-19, wherein the  $\alpha_{1H}$ -subunit is an  $\alpha_{1H-1}$  subunit or an  $\alpha_{1H-2}$  subunit.

21. The eukaryotic cell of claim 20, wherein the  $\alpha_{1H}$  subunit is a human calcium channel subunit.

22. A method for identifying a compound that modulates the activity of a calcium channel that contains an  $\alpha_{1H}$  subunit, comprising; suspending the eukaryotic cell of any of claims 8-21 in a solution containing the compound and a calcium channel selective ion:

- depolarizing the cell membrane of the cell; and detecting the current or ions flowing into the cell,

wherein:

the heterologous calcium channel includes at least one calcium channel subunit encoded by DNA or RNA that is heterologous to the cell,

the current that is detected is different from that produced by depolarizing the same or a substantially identical cell in the presence of the same calcium channel selective ion but in the absence of the compound.

5           23. The method of claim 22, wherein prior to the depolarization step the cell is maintained at a holding potential which substantially inactivates calcium channels that are endogenous to the cell.

          24. The method of claim 23, wherein:

                  the cell is an amphibian oocyte;

10           the heterologous subunits are encoded by nucleic acid injected into the oocyte; and

                  the heterologous subunits include an  $\alpha_{1H}$ -subunit.

          25. The method of claim 24, wherein the subunits encoded by the nucleic acid further comprise a  $\alpha_2\delta$ -subunit.

15           26. The method of any of claims 22-25, wherein the cell is an HEK cell and the heterologous subunit is encoded by heterologous nucleic acid.

          27. The method of any of claims 22-26, wherein the  $\alpha_{1H}$ -subunit is an  $\alpha_{1H-1}$ -subunit or an  $\alpha_{1H-2}$ -subunit.

20           28. The method of claim 22, wherein:

                  the heterologous calcium channel includes at least one calcium channel subunit encoded by DNA or RNA that is heterologous to the cell;

                  at least one subunit is an  $\alpha_{1H}$ -subunit;

                  the current that is detected is different from that produced by  
25   depolarizing the same or a substantially identical cell in the presence of the same calcium channel selective ion but in the absence of the compound.



29. A substantially pure  $\alpha_1$ -subunit encoded by the nucleic acid molecule of any of claims 1-5.

30. An RNA or DNA probe of at least 16 bases in length, comprising at least 16 substantially contiguous nucleic acid bases from  
5 the sequence of nucleotides of claim 1 that encodes an  $\alpha_{1H}$ -subunit of a calcium channel.

31. The probe of claim 28 that contains at least 30 nucleic acid bases that encode the subunit of a calcium channel.

32. A method for identifying nucleic acids that encode a  $\alpha_{1H}$   
10 subunit of a calcium channel subunit, comprising hybridizing under conditions of at least low stringency a probe of claim 28 to a library of nucleic acid fragments;, and selecting hybridizing fragments.

33. The method of claim 30, wherein hybridization is effected under conditions of high stringency.

15 34. A method for identifying cells or tissues that express a calcium channel subunit-encoding nucleic acid, comprising hybridizing under conditions of at least low stringency a probe of claim 30 or claim 31 with mRNA expressed in the cells or tissues or cDNA produced from the mRNA, and thereby identifying cells or tissue that express mRNA that  
20 encodes the subunit.

35. The method of claim 32, wherein hybridization is effected under conditions of high stringency.

36. A method for producing a subunit of a calcium channel, comprising introducing the nucleic acid molecule of any of claims 1-5 into  
25 a host cell, under conditions whereby the encoded subunit is expressed.

37. The method of claim 35, wherein the cell is a eukaryotic cell.

38. A eukaryotic cell, comprising a heterologous calcium channel encoded by nucleic acid encoding an  $\alpha$ -subunit of a calcium channel, wherein the heterologous calcium channel is a low voltage activated channel or a T-type channel.

5        39. The eukaryotic cell of any of claims 6-21 and 38, wherein the  $\alpha$ -subunit comprises the sequence of amino acids set forth in any of SEQ ID Nos. 12-16.

40. An isolated nucleic acid molecule, comprising the sequence of amino acids encoded by nucleotides 1506 to 2627 of SEQ ID No. 12.

10       41. The isolated nucleic acid molecule of claim 40, comprising the sequence of nucleotides set forth in nucleotides 1506 to 2627 of SEQ ID No. 12.

42. The nucleic acid of any of claims 1-5, 40 and 41 that is RNA.

15       43. The nucleic acid of any of claims 1-5, 40 and 41 that is DNA.

44. The cell of claim 8, further comprising nucleic acid that encodes a reporter gene construct containing a reporter gene in operative linkage with one or more transcriptional control elements that is regulated  
20 by a calcium channel.

45. A method for identifying compounds that modulate the activity of a low-voltage activated calcium channel, the method comprising:

25       comparing the difference in the amount of transcription of a the reporter gene in the cell of claim 44 in the presence of the compound with the amount of transcription in the absence of the compound, or with the amount of transcription in the absence of the heterologous calcium channel, whereby compounds that

modulate the activity of the heterologous calcium channel in the cell are identified.

46. The nucleic acid molecule of any of claims 1-5, 40 and 41, wherein the calcium channel is a human calcium channel.

5        47. A screening assay for identifying a compound that modulates the activity of a low-voltage activated (LVA) calcium channel comprising the steps of:

          contacting the test compound with a cell that expresses a LVA calcium channel; and

10        measuring the activity of the LVA channel in the cell before and after the addition of the test compound or in comparable cell that does not express the LVA channel; and

          determining that the test compound modulates the activity of the low-voltage calcium channel if the measurement after compound addition  
15        is different from the measurement before the compound addition or if the measurement in presence of the receptor is different from the measurement in the absence of the receptor.

          48. The method of claim 47, wherein the LVA channel is produced by introducing the a nucleic acid that encodes the LVA into the  
20        cell under conditions whereby the encoded LVA is expressed.

          49. The method of claim 47 or claim 48, wherein the LVA is a T-type channel.

          50. The method of any of claims 47-49, wherein the LVA comprises an  $\alpha_{1H}$ -subunit of a calcium channel.

25        51. The method of any of claims 47-50, wherein the cell expresses a low-voltage calcium channel having a relative conductance of  $Ba^{2+}$  of about 5 pS to about 9 pS, an activation time of about 2 to about 8 milliseconds, a kinetics of activation  $V_{1/2}$  value of about -60 millivolts to

about 26 millivolts, an inactivation time of about 10 to about 30 milliseconds, a kinetics of inactivation  $V_{1/2}$  value of about -100 millivolts to about -500 millivolts, and a tail deactivation time of about 2 to about 12 milliseconds.

5            52. The screening method of any of claims 47-51, wherein the isolated nucleic acid molecule comprises a sequence of nucleotides encoding an  $\alpha_{1H}$ -subunit of a calcium channel.

53. A compound identified by the method of any of claims 45 and 47-52.

10           54. A method of identifying compounds for treatment of LVA-type calcium channel mediated disorders, comprising identifying compounds that modulate the activity of LVA-type channels in cells that express channels containing a subunit encoded by the nucleic acid of any of claims 1-5, 40 and 41.

15           55. Compounds identified by the method of 54.

56. The method of claim 54, wherein the channels are produced by introduction of the nucleic acid of any of claims 1-5, 40 and 41 into cells under conditions whereby channels that contain the encoded subunit are expressed.

20           57. The method of claim 54 or claim 56, wherein the disorder is selected from among, neurological, endocrinological, cardiovascular, urological, hepatic, respiratory, and vascular disorders.

FIGURE 1

Steady-state activation and inactivation

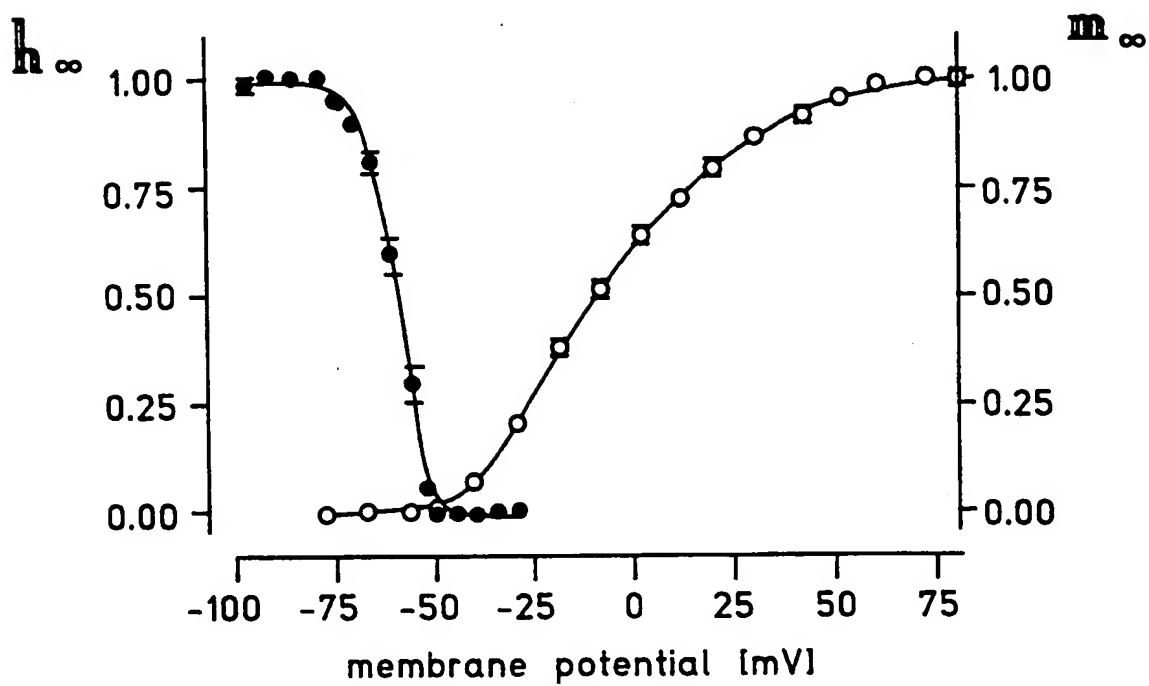


FIGURE 2A

Kinetics of activation

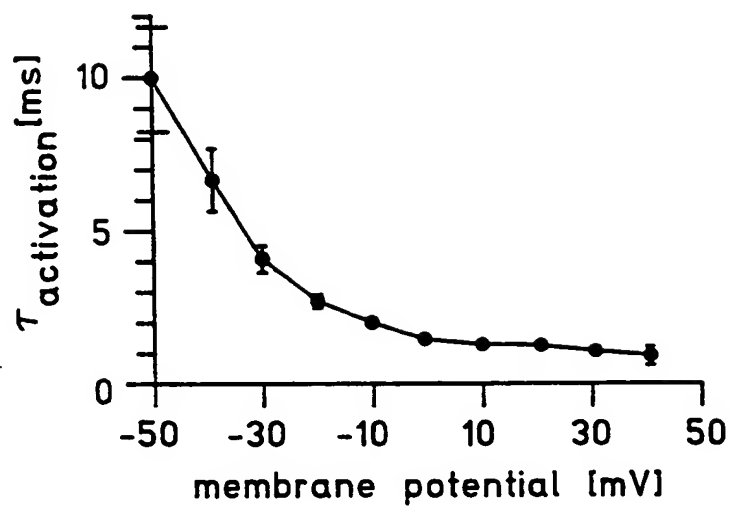


FIGURE 2B

Kinetics of inactivation

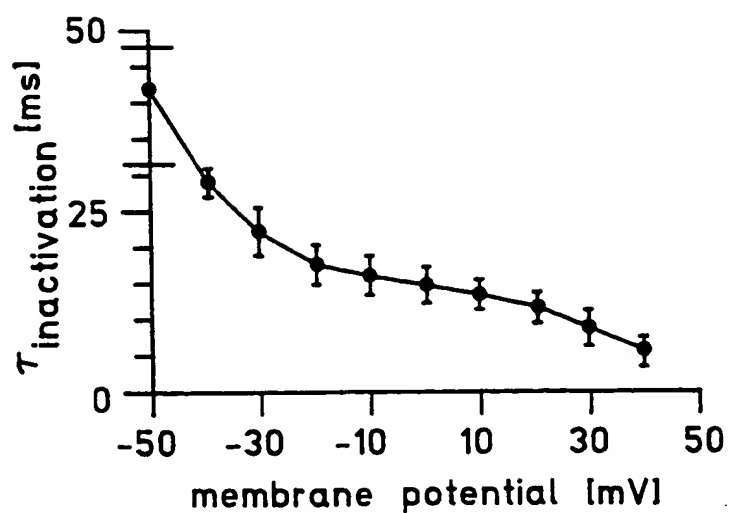
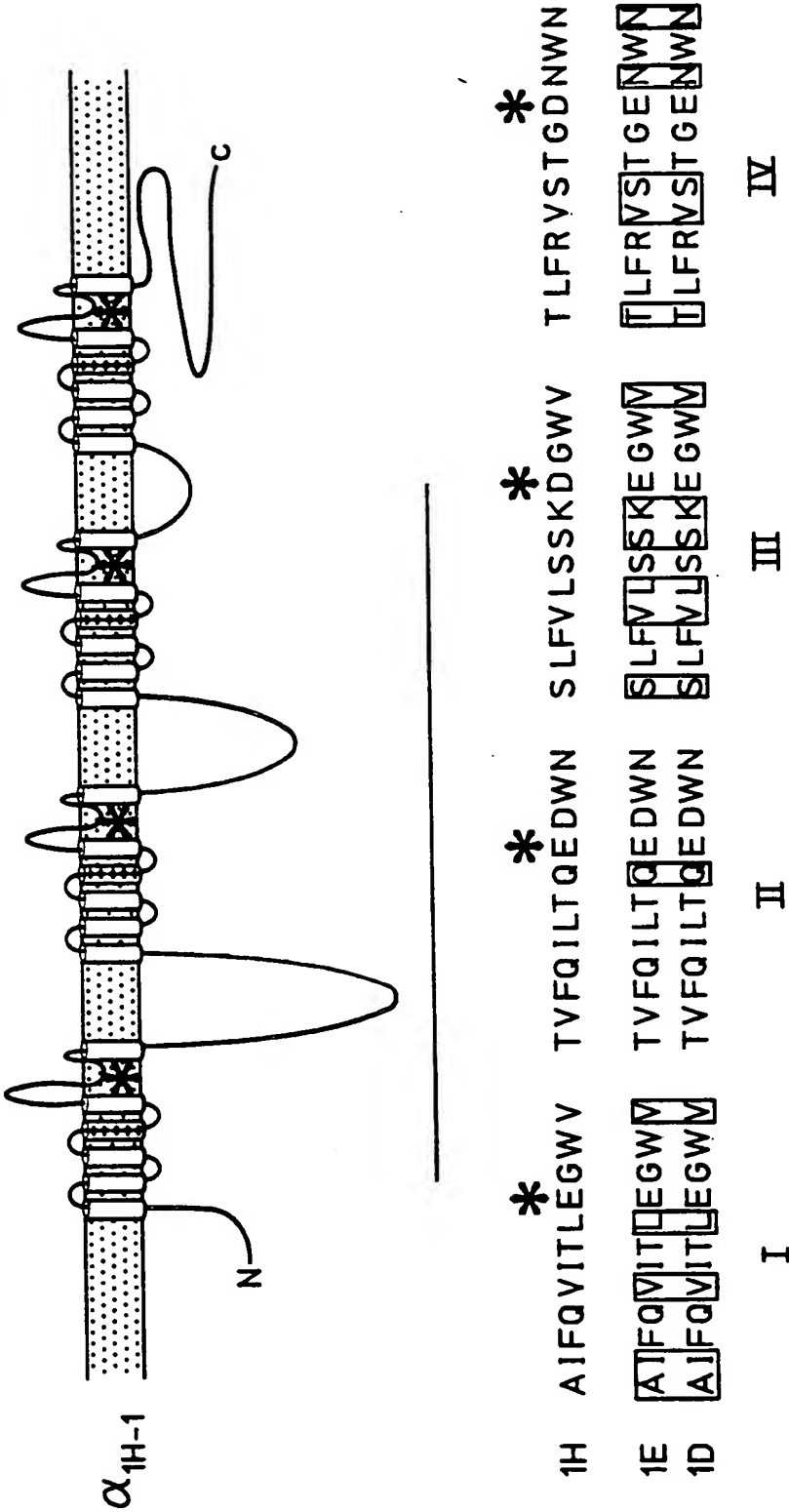


FIGURE 3  
Features of the  $\alpha_{1H}$  Subunit



## Tail current deactivation

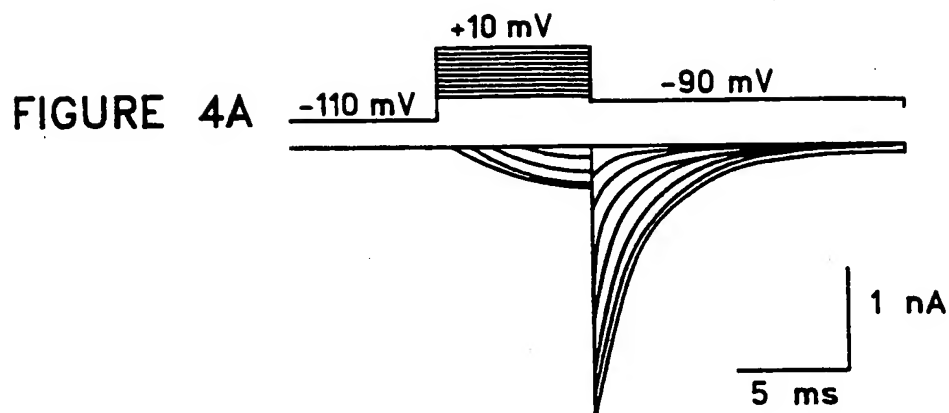


FIGURE 4B

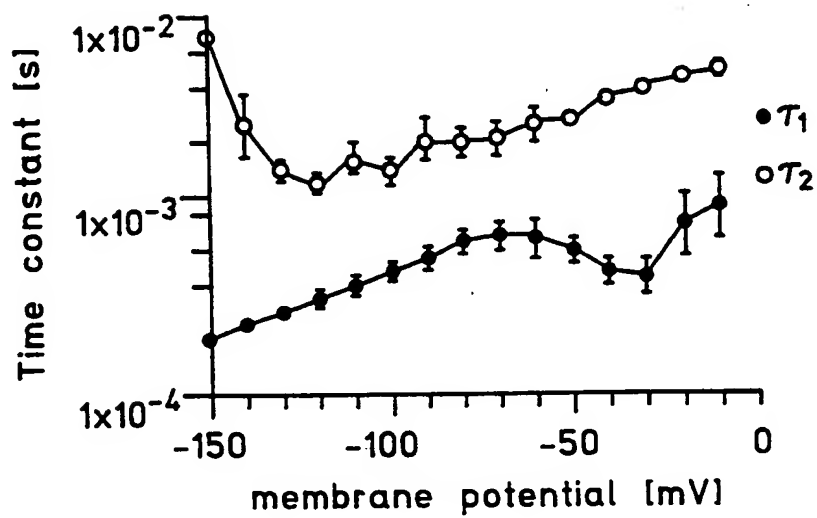
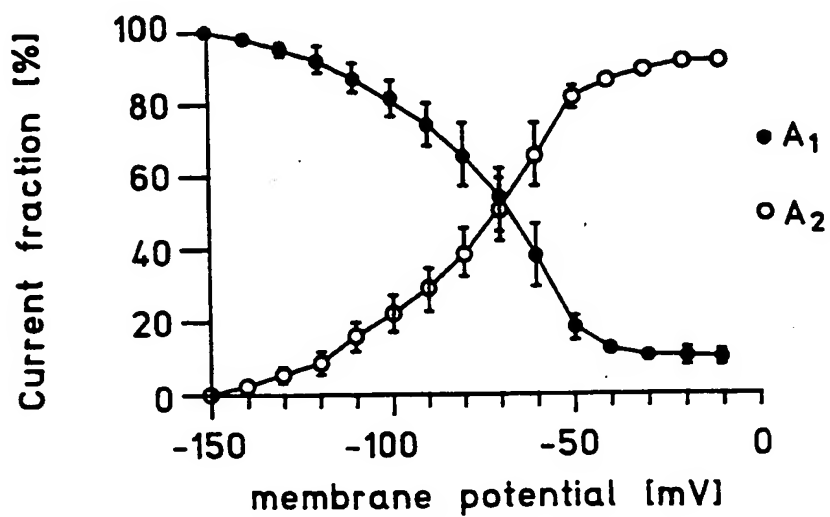


FIGURE 4C





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(F) POSTAL CODE (ZIP): 92007

(ii) TITLE OF INVENTION: CALCIUM CHANNEL COMPOSITIONS AND METHODS

(iii) NUMBER OF SEQUENCES: 16

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: DOS  
(D) SOFTWARE: FastSEQ Version 1.5 and PatentIn 2.0

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(A) APPLICATION NUMBER: 08/984,709  
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: SIBIA Neurosciences, Inc.  
(B) STREET: 505 Coast Boulevard South, Suite 300  
(C) CITY: La Jolla  
(D) STATE: California  
(E) COUNTRY: US  
(F) POSTAL CODE (ZIP): 92037-4641

## (i) INVENTOR/APPLICANT:

(A) NAME: Mark E. Williams  
(B) STREET: 946 Jasmine Court  
(C) CITY: Carlsbad  
(D) STATE: California  
(E) COUNTRY: USA  
(F) POSTAL CODE (ZIP): 92009

## (i) INVENTOR/APPLICANT:

(A) NAME: Kenneth A. Stauderman  
(B) STREET: 3615 Lotus Dr.  
(C) CITY: San Diego  
(D) STATE: California  
(E) COUNTRY: USA  
(F) POSTAL CODE (ZIP): 92106

## (i) INVENTOR/APPLICANT:

(A) NAME: Michael M. Harpold  
(B) STREET: 1462 Encina Road  
(C) CITY: Sante Fe  
(D) STATE: New Mexico  
(E) COUNTRY: USA  
(F) POSTAL CODE (ZIP): 87505-4726

## (i) INVENTOR/APPLICANT:

(A) NAME: Michael Hans  
(B) STREET: 2635 Clemente Terrace  
(C) CITY: San Diego  
(D) STATE: California  
(E) COUNTRY: USA  
(F) POSTAL CODE (ZIP): 92122

## (i) INVENTOR/APPLICANT:

(A) NAME: Arturo Urrutia  
(B) STREET: 778 Beech Avenue  
(C) CITY: Chula Vista  
(D) STATE: California  
(E) COUNTRY: USA  
(F) POSTAL CODE (ZIP): 91910

## (i) INVENTOR/APPLICANT:

(A) NAME: Mark S. Washburn  
(B) STREET: 1535 Kings Cross Drive  
(C) CITY: Cardiff  
(D) STATE: California

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TYCCCTTGAA GAGCTGNACC CC

22

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 17 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGTGCACGTC ACGCTAG

17

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AATTCTAGCG TGACGTGCAC G

21

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACNGTGTTYC AGATCCTGAC

2

(2) INFORMATION FOR SEQ ID NO:5:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATCCTGACNG GNGARGACTG GAA

23

(2) INFORMATION FOR SEQ ID NO:6:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TYCCCTTGAA GAGCTGNACN GC

22

(2) INFORMATION FOR SEQ ID NO:7:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TYCCCTTGA AGAGCTGNAC CCC

22

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AACTGYATYA CCCTGGC

17

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATYACCTTGG CNATGGAGCG

20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GARATGATGA TGAARGT

17

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 342 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

GGGAGATGAT GGTGAAAGTG GTGGCCCTGG GGCTGCTGTC CGGCGAGCAC GCCTACCTGC      60
AGAGCAGCTG GAACCTGCTG GATGGGCTGC TGGTGCTGGT GTCCCTGGTG GACATTGTCTG    120
TGGCCATGGC CTCGGCTGGT GCGGCCAAGA TCCTGGGTGT TCTGCGCGTG CTGCGTCTGC      180
TGGCGACCCT GCGGCCTCTG AGGGTCATCA GCCGGGCCCC GGGCCTCAAG CTGGTGGTGG      240
AGACGCTGAT ATCATCACTC AGGCCCATTG GGAACATCGT CCTCATCTGC TGGCGCTTCT      300
TCATCATTTT TGGCATTTTG GGGGTTTCAGC TCTTCAAGGG      340

```

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7898 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 249...7307

(D) OTHER INFORMATION:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

CGAGGCCGCC GCCGTGCCT CCGCCGGGCG AGCCGGAGCC GGAGTCGAGC CGCGGCCGGG      60
AGCCGGGCGG GCTGGGGACG CCGGCCGGGG GCGGAGGCGC TGGGGGCCGG GGCCGGGGCC      120
GGGGGCCGAG GCGCTGGGGG CCGGGGCCGG GGCCGGGCGC CGAGCGGGGT CCGCGGTGAC      180
CGCGCCGCCC GGGCGATGCC CGCGGGGACG CCGCCGGCCA GCAGAGCGAG GTGCTGCCCG      240
CCGCCACC ATG ACC GAG GGC GCA CGG GCC GCC GAC GAG GTC CGG GTG CCC      290
      Met Thr Glu Gly Ala Arg Ala Ala Asp Glu Val Arg Val Pro
          1             5             10

CTG GGC GCG CCG CCC CCT GGC CCT GCG GCG TTG GTG GGG GCG TCC CCG      338
Leu Gly Ala Pro Pro Pro Gly Pro Ala Ala Leu Val Gly Ala Ser Pro
15             20             25             30

```

GAG AGC CCC GGG GCG CCG GGA CGC GAG GCG GAG CGG GGG TCC GAG CTC	386
Glu Ser Pro Gly Ala Pro Gly Arg Glu Ala Glu Arg Gly Ser Glu Leu	
35 40 45	
GGC GTG TCA CCC TCC GAG AGC CCG GCG GCC GAG CGC GGC GCG GAG CTG	434
Gly Val Ser Pro Ser Glu Ser Pro Ala Ala Glu Arg Gly Ala Glu Leu	
50 55 60	
GGT GCC GAC GAG GAG CAG CGC GTC CCG TAC CCG GCC TTG GCG GCC ACG	482
Gly Ala Asp Glu Glu Gln Arg Val Pro Tyr Pro Ala Leu Ala Ala Thr	
65 70 75	
GTC TTC TTC TGC CTC GGT CAG ACC ACG CGG CCG CGC AGC TGG TGC CTC	530
Val Phe Phe Cys Leu Gly Gln Thr Thr Arg Pro Arg Ser Trp Cys Leu	
80 85 90	
CGG CTG GTC TGC AAC CCA TGG TTC GAG CAC GTG AGC ATG CTG GTA ATC	578
Arg Leu Val Cys Asn Pro Trp Phe Glu His Val Ser Met Leu Val Ile	
95 100 105 110	
ATG CTC AAC TGC GTG ACC CTG GGC ATG TTC CGG CCC TGT GAG GAC GTT	626
Met Leu Asn Cys Val Thr Leu Gly Met Phe Arg Pro Cys Glu Asp Val	
115 120 125	
GAG TGC GGC TCC GAG CGC TGC AAC ATC CTG GAG GCC TTT GAC GCC TTC	674
Glu Cys Gly Ser Glu Arg Cys Asn Ile Leu Glu Ala Phe Asp Ala Phe	
130 135 140	
ATT TTC GCC TTT TTT GCG GTG GAG ATG GTC ATC AAG ATG GTG GCC TTG	722
Ile Phe Ala Phe Phe Ala Val Glu Met Val Ile Lys Met Val Ala Leu	
145 150 155	
GGG CTG TTC GGG CAG AAG TGT TAC CTG GGT GAC ACG TGG AAC AGG CTG	770
Gly Leu Phe Gly Gln Lys Cys Tyr Leu Gly Asp Thr Trp Asn Arg Leu	
160 165 170	
GAT TTC TTC ATC GTC GTG GCG GGC ATG ATG GAG TAC TCG TTG GAC GGA	818
Asp Phe Phe Ile Val Val Ala Gly Met Met Glu Tyr Ser Leu Asp Gly	
175 180 185 190	
CAC AAC GTG AGC CTC TCG GCT ATC AGG ACC GTG CGG GTG CTG CGG CCC	866
His Asn Val Ser Leu Ser Ala Ile Arg Thr Val Arg Val Leu Arg Pro	
195 200 205	
CTC CGC GCC ATC AAC CGC GTG CCT AGC ATG CGG ATC CTG GTC ACT CTG	914
Leu Arg Ala Ile Asn Arg Val Pro Ser Met Arg Ile Leu Val Thr Leu	
210 215 220	
CTG CTG GAT ACG CTG CCC ATG CTC GGG AAC GTC CTT CTG CTG TGC TTC	962
Leu Leu Asp Thr Leu Pro Met Leu Gly Asn Val Leu Leu Leu Cys Phe	
225 230 235	
TTC GTC TTC TTC ATT TTC GGC ATC GTT GGC GTC CAG CTC TGG GCT GGC	1010
Phe Val Phe Phe Ile Phe Gly Ile Val Gly Val Gln Leu Trp Ala Gly	
240 245 250	
CTC CTG CGG AAC CGC TGC TTC CTG GAC AGT GCC TTT GTC AGG AAC AAC	1058
Leu Leu Arg Asn Arg Cys Phe Leu Asp Ser Ala Phe Val Arg Asn Asn	

255											260											265											270																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																														
AAC	CTG	ACC	TTC	CTG	CGG	CCG	TAC	TAC	CAG	ACG	GAG	GAG	GGC	GAG	GAG																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																



GTG	CAA	GGC	CAG	GGT	CCC	GGG	CAC	CGC	CAG	CGC	CGG	GCA	GGC	AGG	CAC	1778
Val	Gln	Gly	Gln	Gly	Pro	Gly	His	Arg	Gln	Arg	Arg	Ala	Gly	Arg	His	
495					500					505					510	
ACA	GCC	TCG	GTG	CAC	CAC	CTG	GTC	TAC	CAC	CAC	CAT	CAC	CAC	CAC	CAC	1826
Thr	Ala	Ser	Val	His	His	Leu	Val	Tyr	His	His	His	His	His	His	His	
				515					520					525		
CAC	CAC	TAC	CAT	TTC	AGC	CAT	GGC	AGC	CCC	CGC	AGG	CCC	GGC	CCC	GAG	1874
His	His	Tyr	His	Phe	Ser	His	Gly	Ser	Pro	Arg	Arg	Pro	Gly	Pro	Glu	
			530					535					540			
CCA	GGC	GCC	TGC	GAC	ACC	AGG	CTG	GTC	CGA	GCT	GGC	GCG	CCC	CCC	TCG	1922
Pro	Gly	Ala	Cys	Asp	Thr	Arg	Leu	Val	Arg	Ala	Gly	Ala	Pro	Pro	Ser	
		545					550					555				
CCA	CCT	TCC	CCA	GGC	CGC	GGA	CCC	CCC	GAC	GCA	GAG	TCT	GTG	CAC	AGC	1970
Pro	Pro	Ser	Pro	Gly	Arg	Gly	Pro	Pro	Asp	Ala	Glu	Ser	Val	His	Ser	
	560					565					570					
ATC	TAC	CAT	GCC	GAC	TGC	CAC	ATA	GAG	GGG	CCG	CAG	GAG	AGG	GCC	CGG	2018
Ile	Tyr	His	Ala	Asp	Cys	His	Ile	Glu	Gly	Pro	Gln	Glu	Arg	Ala	Arg	
575					580					585					590	
GTG	GCA	CAT	GCC	GCA	GCC	ACT	GCC	GCT	GCC	AGC	CTC	AGG	CTG	GCC	ACA	2066
Val	Ala	His	Ala	Ala	Ala	Thr	Ala	Ala	Ala	Ser	Leu	Arg	Leu	Ala	Thr	
				595					600					605		
GGG	CTG	GGC	ACC	ATG	AAC	TAC	CCC	ACG	ATC	CTG	CCC	TCA	GGG	GTG	GGC	2114
Gly	Leu	Gly	Thr	Met	Asn	Tyr	Pro	Thr	Ile	Leu	Pro	Ser	Gly	Val	Gly	
			610					615					620			
AGC	GGC	AAA	GGC	AGC	ACC	AGC	CCC	GGA	CCC	AAG	GGG	AAG	TGG	GCC	GGT	2162
Ser	Gly	Lys	Gly	Ser	Thr	Ser	Pro	Gly	Pro	Lys	Gly	Lys	Trp	Ala	Gly	
		625					630					635				
GGA	CCG	CCA	GGC	ACC	GGG	GGG	CAC	GGC	CCG	TTG	AGC	TTG	AAC	AGC	CCT	2210
Gly	Pro	Pro	Gly	Thr	Gly	Gly	His	Gly	Pro	Leu	Ser	Leu	Asn	Ser	Pro	
	640					645					650					
GAT	CCC	TAC	GAG	AAG	ATC	CCG	CAT	GTG	GTC	GGG	GAG	CAT	GGA	CTG	GGC	2258
Asp	Pro	Tyr	Glu	Lys	Ile	Pro	His	Val	Val	Gly	Glu	His	Gly	Leu	Gly	
655					660					665					670	
CAG	GCC	CCT	GGC	CAT	CTG	TCG	GGC	CTC	AGT	GTG	CCC	TGC	CCC	CTG	CCC	2306
Gln	Ala	Pro	Gly	His	Leu	Ser	Gly	Leu	Ser	Val	Pro	Cys	Pro	Leu	Pro	
				675					680					685		
AGC	CCC	CCA	GCG	GGC	ACA	CTG	ACC	TGT	GAG	CTG	AAG	AGC	TGC	CCG	TAC	2354
Ser	Pro	Pro	Ala	Gly	Thr	Leu	Thr	Cys	Glu	Leu	Lys	Ser	Cys	Pro	Tyr	
			690					695					700			
TGC	ACC	CGT	GCC	CTG	GAG	GAC	CCG	GAG	GGT	GAG	CTC	AGC	GGC	TCG	GAA	2402
Cys	Thr	Arg	Ala	Leu	Glu	Asp	Pro	Glu	Gly	Glu	Leu	Ser	Gly	Ser	Glu	
		705					710					715				
AGT	GGA	GAC	TCA	GAT	GGC	CGT	GGC	GTC	TAT	GAA	TTC	ACG	CAG	GAC	GTC	2450
Ser	Gly	Asp	Ser	Asp	Gly	Arg	Gly	Val	Tyr	Glu	Phe	Thr	Gln	Asp	Val	

720	725	730	
CGG CAC GGT GAC CGC TGG GAC CCC ACG CGA CCA CCC CGT GCG ACG GAC Arg His Gly Asp Arg Trp Asp Pro Thr Arg Pro Pro Arg Ala Thr Asp 735 740 745 750			2498
ACA CCA GGC CCA GGC CCA GGC AGC CCC CAG CGG CGG GCA CAG CAG AGG Thr Pro Gly Pro Gly Pro Gly Ser Pro Gln Arg Arg Ala Gln Gln Arg 755 760 765			2546
GCA GCC CCG GGC GAG CCA GGC TGG ATG GGC CGC CTC TGG GTT ACC TTC Ala Ala Pro Gly Glu Pro Gly Trp Met Gly Arg Leu Trp Val Thr Phe 770 775 780			2594
AGC GGC AAG CTG CGC CGC ATC GTG GAC AGC AAG TAC TTC AGC CGT GGC Ser Gly Lys Leu Arg Arg Ile Val Asp Ser Lys Tyr Phe Ser Arg Gly 785 790 795			2642
ATC ATG ATG GCC ATC CTT GTC AAC ACG CTG AGC ATG GGC GTG GAG TAC Ile Met Met Ala Ile Leu Val Asn Thr Leu Ser Met Gly Val Glu Tyr 800 805 810			2690
CAT GAG CAG CCC GAG GAG CTG ACT AAT GCT CTG GAG ATC AGC AAC ATC His Glu Gln Pro Glu Glu Leu Thr Asn Ala Leu Glu Ile Ser Asn Ile 815 820 825 830			2738
GTG TTC ACC AGC ATG TTT GCC CTG GAG ATG CTG CTG AAG CTG CTG GCC Val Phe Thr Ser Met Phe Ala Leu Glu Met Leu Leu Lys Leu Leu Ala 835 840 845			2786
TGC GGC CCT CTG GGC TAC ATC CGG AAC CCG TAC AAC ATC TTC GAC GGC Cys Gly Pro Leu Gly Tyr Ile Arg Asn Pro Tyr Asn Ile Phe Asp Gly 850 855 860			2834
ATC ATC GTG GTC ATC AGC GTC TGG GAG ATC GTG GGG CAG GCG GAC GGT Ile Ile Val Val Ile Ser Val Trp Glu Ile Val Gly Gln Ala Asp Gly 865 870 875			2882
GGC TTG TCT GTG CTG CGC ACC TTC CGG CTG CTG CGT GTG CTG AAG CTG Gly Leu Ser Val Leu Arg Thr Phe Arg Leu Leu Arg Val Leu Lys Leu 880 885 890			2930
GTG CGC TTT CTG CCA GCC CTG CGG CGC CAG CTC GTG GTG CTG GTG AAG Val Arg Phe Leu Pro Ala Leu Arg Arg Gln Leu Val Val Leu Val Lys 895 900 905 910			2978
ACC ATG GAC AAC GTG GCT ACC TTC TGC ACG CTG CTC ATG CTC TTC ATT Thr Met Asp Asn Val Ala Thr Phe Cys Thr Leu Leu Met Leu Phe Ile 915 920 925			3026
TTC ATC TTC AGC ATC CTG GGC ATG CAC CTT TTC GGC TGC AAG TTC AGC Phe Ile Phe Ser Ile Leu Gly Met His Leu Phe Gly Cys Lys Phe Ser 930 935 940			3074
CTG AAG ACA GAC ACC GGA GAC ACC GTG CCT GAC AGG AAG AAC TTC GAC Leu Lys Thr Asp Thr Gly Asp Thr Val Pro Asp Arg Lys Asn Phe Asp 945 950 955			3122

TCC CTG CTG TGG GCC ATC GTC ACC GTG TTC CAG ATC CTG ACC CAG GAG Ser Leu Leu Trp Ala Ile Val Thr Val Phe Gln Ile Leu Thr Gln Glu 960 965 970	3170
GAC TGG AAC GTG GTC CTG TAC AAC GGC ATG GCC TCC ACC TCC TCC TGG Asp Trp Asn Val Val Leu Tyr Asn Gly Met Ala Ser Thr Ser Ser Trp 975 980 985 990	3218
GCC GCC CTC TAC TTC GTG GCC CTC ATG ACC TTC GGC AAC TAT GTG CTC Ala Ala Leu Tyr Phe Val Ala Leu Met Thr Phe Gly Asn Tyr Val Leu 995 1000 1005	3266
TTC AAC CTG CTG GTG GCC ATC CTC GTG GAG GGC TTC CAG GCG GAG GGC Phe Asn Leu Leu Val Ala Ile Leu Val Glu Gly Phe Gln Ala Glu Gly 1010 1015 1020	3314
GAT GCC AAC AGA TCC GAC ACG GAC GAG GAC AAG ACG TCG GTC CAC TTC Asp Ala Asn Arg Ser Asp Thr Asp Glu Asp Lys Thr Ser Val His Phe 1025 1030 1035	3362
GAG GAG GAC TTC CAC AAG CTC AGA GAA CTC CAG ACC ACA GAG CTG AAG Glu Glu Asp Phe His Lys Leu Arg Glu Leu Gln Thr Thr Glu Leu Lys 1040 1045 1050	3410
ATG TGT TCC CTG GCC GTG ACC CCC AAC GGG CAC CTG GAG GGA CGA GGC Met Cys Ser Leu Ala Val Thr Pro Asn Gly His Leu Glu Gly Arg Gly 1055 1060 1065 1070	3458
AGC CTG TCC CCT CCC CTC ATC ATG TGC ACA GCT GCC ACG CCC ATG CCT Ser Leu Ser Pro Pro Leu Ile Met Cys Thr Ala Ala Thr Pro Met Pro 1075 1080 1085	3506
ACC CCC AAG AGC TCA CCA TTC CTG GAT GCA GCC CCC AGC CTC CCA GAC Thr Pro Lys Ser Ser Pro Phe Leu Asp Ala Ala Pro Ser Leu Pro Asp 1090 1095 1100	3554
TCT CGG CGT GGC AGC AGC AGC TCC GGG GAC CCG CCA CTG GGA GAC CAG Ser Arg Arg Gly Ser Ser Ser Ser Gly Asp Pro Pro Leu Gly Asp Gln 1105 1110 1115	3602
AAG CCT CCG GCC AGC CTC CGA AGT TCT CCC TGT GCC CCC TGG GGC CCC Lys Pro Pro Ala Ser Leu Arg Ser Ser Pro Cys Ala Pro Trp Gly Pro 1120 1125 1130	3650
AGT GGC GCC TGG AGC AGC CGG CGC TCC AGC TGG AGC AGC CTG GGC CGT Ser Gly Ala Trp Ser Ser Arg Arg Ser Ser Trp Ser Ser Leu Gly Arg 1135 1140 1145 1150	3698
GCC CCC AGC CTC AAG CGC CGC GGC CAG TGT GGG GAA CGT GAG TCC CTG Ala Pro Ser Leu Lys Arg Arg Gly Gln Cys Gly Glu Arg Glu Ser Leu 1155 1160 1165	3746
CTG TCT GGC GAG GGC AAG GGC AGC ACC GAC GAC GAA GCT GAG GAC GGC Leu Ser Gly Glu Gly Lys Gly Ser Thr Asp Asp Glu Ala Glu Asp Gly 1170 1175 1180	3794
AGG GCC GCG CCC GGG CCC CGT GCC ACC CCA CTG CGG CGG GCC GAG TCC Arg Ala Ala Pro Gly Pro Arg Ala Thr Pro Leu Arg Arg Ala Glu Ser	3842

1185	1190	1195	
CTG GAC CCA CGG CCC CTG CGG CCG GCC GCC CTC CCG CCT ACC AAG TGC Leu Asp Pro Arg Pro Leu Arg Pro Ala Ala Leu Pro Pro Thr Lys Cys 1200 1205 1210			3890
CGC GAT CGC GAC GGG CAG GTG GTG GCC CTG CCC AGC GAC TTC TTC CTG Arg Asp Arg Asp Gly Gln Val Val Ala Leu Pro Ser Asp Phe Phe Leu 1215 1220 1225 1230			3938
CGC ATC GAC AGC CAC CGT GAG GAT GCA GCC GAG CTT GAC GAC GAC TCG Arg Ile Asp Ser His Arg Glu Asp Ala Ala Glu Leu Asp Asp Asp Ser 1235 1240 1245			3986
GAG GAC AGC TGC TGC CTC CGC CTG CAT AAA GTG CTG GAG CCC TAC AAG Glu Asp Ser Cys Cys Leu Arg Leu His Lys Val Leu Glu Pro Tyr Lys 1250 1255 1260			4034
CCC CAG TGG TGC CGG AGC CGC GAG GCC TGG GCC CTC TAC CTC TTC TCC Pro Gln Trp Cys Arg Ser Arg Glu Ala Trp Ala Leu Tyr Leu Phe Ser 1265 1270 1275			4082
CCA CAG AAC CGG TTC CGC GTC TCC TGC CAG AAG GTC ATC ACA CAC AAG Pro Gln Asn Arg Phe Arg Val Ser Cys Gln Lys Val Ile Thr His Lys 1280 1285 1290			4130
ATG TTT GAT CAC GTG GTC CTC GTC TTC ATC TTC CTC AAC TGC GTC ACC Met Phe Asp His Val Val Leu Val Phe Ile Phe Leu Asn Cys Val Thr 1295 1300 1305 1310			4178
ATC GCC CTG GAG AGG CCT GAC ATT GAC CCC GGC AGC ACC GAG CGG GTC Ile Ala Leu Glu Arg Pro Asp Ile Asp Pro Gly Ser Thr Glu Arg Val 1315 1320 1325			4226
TTC CTC AGC GTC TCC AAT TAC ATC TTC ACG GCC ATC TTC GTG GCG GAG Phe Leu Ser Val Ser Asn Tyr Ile Phe Thr Ala Ile Phe Val Ala Glu 1330 1335 1340			4274
ATG ATG GTG AAG GTG GTG GCC CTG GGG CTG CTG TCC GGC GAG CAC GCC Met Met Val Lys Val Val Ala Leu Gly Leu Leu Ser Gly Glu His Ala 1345 1350 1355			4322
TAC CTG CAG AGC AGC TGG AAC CTG CTG GAT GGG CTG CTG GTG CTG GTG Tyr Leu Gln Ser Ser Trp Asn Leu Leu Asp Gly Leu Leu Val Leu Val 1360 1365 1370			4370
TCC CTG GTG GAC ATT GTC GTG GCC ATG GCC TCG GCT GGT GGC GCC AAG Ser Leu Val Asp Ile Val Val Ala Met Ala Ser Ala Gly Gly Ala Lys 1375 1380 1385 1390			4418
ATC CTG GGT GTT CTG CGC GTG CTG CGT CTG CTG CGG ACC CTG CGG CCT Ile Leu Gly Val Leu Arg Val Leu Arg Leu Leu Arg Thr Leu Arg Pro 1395 1400 1405			4466
CTA AGG GTC ATC AGC CGG GCC CCG GGC CTC AAG CTG GTG GTG GAG ACG Leu Arg Val Ile Ser Arg Ala Pro Gly Leu Lys Leu Val Val Glu Thr 1410 1415 1420			4514

CTG ATA TCG TCG CTC AGG CCC ATT GGG AAC ATC GTC CTC ATC TGC TGC	4562
Leu Ile Ser Ser Leu Arg Pro Ile Gly Asn Ile Val Leu Ile Cys Cys	
1425 1430 1435	
GCC TTC TTC ATC ATT TTT GGC ATC TTG GGT GTG CAG CTC TTC AAA GGG	4610
Ala Phe Phe Ile Ile Phe Gly Ile Leu Gly Val Gln Leu Phe Lys Gly	
1440 1445 1450	
AAG TTC TAC TAC TGC GAG GGC CCC GAC ACC AGG AAC ATC TCC ACC AAG	4658
Lys Phe Tyr Tyr Cys Glu Gly Pro Asp Thr Arg Asn Ile Ser Thr Lys	
1455 1460 1465 1470	
GCA CAG TGC CGG GCC GCC CAC TAC CGC TGG GTG CGA CGC AAG TAC AAC	4706
Ala Gln Cys Arg Ala Ala His Tyr Arg Trp Val Arg Arg Lys Tyr Asn	
1475 1480 1485	
TTC GAC AAC CTG GGC CAG GCC CTG ATG TCG CTG TTC GTG CTG TCA TCC	4754
Phe Asp Asn Leu Gly Gln Ala Leu Met Ser Leu Phe Val Leu Ser Ser	
1490 1495 1500	
AAG GAT GGA TGG GTG AAC ATC ATG TAC GAC GGG CTG GAT GCC GTG GGT	4802
Lys Asp Gly Trp Val Asn Ile Met Tyr Asp Gly Leu Asp Ala Val Gly	
1505 1510 1515	
GTC GAC CAG CAG CCT GTG CAG AAC CAC AAC CCC TGG ATG CTG CTG TAC	4850
Val Asp Gln Gln Pro Val Gln Asn His Asn Pro Trp Met Leu Leu Tyr	
1520 1525 1530	
TTC ATC TCC TTC CTG CTC ATC GTC AGC TTC TTC GTG CTC AAC ATG TTC	4898
Phe Ile Ser Phe Leu Leu Ile Val Ser Phe Phe Val Leu Asn Met Phe	
1535 1540 1545 1550	
GTG GGC GTC GTG GTC GAG AAC TTC CAC AAG TGC CGG CAG CAC CAG GAG	4946
Val Gly Val Val Val Glu Asn Phe His Lys Cys Arg Gln His Gln Glu	
1555 1560 1565	
GCG GAG GAG GCG CGG CGG CGA GAG GAG AAG CGG CTG CGG CGC CTA GAG	4994
Ala Glu Glu Ala Arg Arg Arg Glu Glu Lys Arg Leu Arg Arg Leu Glu	
1570 1575 1580	
AGG AGG CGC AGG AGC ACT TTC CCC AGC CCA GAG GCC CAG CGC CGG CCC	5042
Arg Arg Arg Arg Ser Thr Phe Pro Ser Pro Glu Ala Gln Arg Arg Pro	
1585 1590 1595	
TAC TAT GCC GAC TAC TCG CCC ACG CGC CGC TCC ATT CAC TCG CTG TGC	5090
Tyr Tyr Ala Asp Tyr Ser Pro Thr Arg Arg Ser Ile His Ser Leu Cys	
1600 1605 1610	
ACC AGC CAC TAT CTC GAC CTC TTC ATC ACC TTC ATC ATC TGT GTC AAC	5138
Thr Ser His Tyr Leu Asp Leu Phe Ile Thr Phe Ile Ile Cys Val Asn	
1615 1620 1625 1630	
GTC ATC ACC ATG TCC ATG GAG CAC TAT AAC CAA CCC AAG TCG CTG GAC	5186
Val Ile Thr Met Ser Met Glu His Tyr Asn Gln Pro Lys Ser Leu Asp	
1635 1640 1645	
GAG GCC CTC AAG TAC TGC AAC TAC GTC TTC ACC ATC GTG TTT GTC TTC	5234
Glu Ala Leu Lys Tyr Cys Asn Tyr Val Phe Thr Ile Val Phe Val Phe	

1650	1655	1660	
GAG GCT GCA CTG AAG CTG GTA GCA TTT GGG TTC CGT CGG TTC TTC AAG Glu Ala Ala Leu Lys Leu Val Ala Phe Gly Phe Arg Arg Phe Phe Lys 1665 1670 1675			5282
GAC AGG TGG AAC CAG CTG GAC CTG GCC ATC GTG CTG CTG TCA CTC ATG Asp Arg Trp Asn Gln Leu Asp Leu Ala Ile Val Leu Leu Ser Leu Met 1680 1685 1690			5330
GGC ATC ACG CTG GAG GAG ATA GAG ATG AGC GCC GCG CTG CCC ATC AAC Gly Ile Thr Leu Glu Glu Ile Glu Met Ser Ala Ala Leu Pro Ile Asn 1695 1700 1705 1710			5378
CCC ACC ATC ATC CGC ATC ATG CGC GTG CTT CGC ATT GCC CGT GTG CTG Pro Thr Ile Ile Arg Ile Met Arg Val Leu Arg Ile Ala Arg Val Leu 1715 1720 1725			5426
AAG CTG CTG AAG ATG GCT ACG GGC ATG CGC GCC CTG CTG GAC ACT GTG Lys Leu Leu Lys Met Ala Thr Gly Met Arg Ala Leu Leu Asp Thr Val 1730 1735 1740			5474
GTG CAA GCT CTC CCC CAG GTG GGG AAC CTG GGC CTT CTT TTC ATG CTC Val Gln Ala Leu Pro Gln Val Gly Asn Leu Gly Leu Leu Phe Met Leu 1745 1750 1755			5522
CTG TTT TTT ATC TAT GCT GCG CTG GGA GTG GAG CTG TTC GGG AGG CTG Leu Phe Phe Ile Tyr Ala Ala Leu Gly Val Glu Leu Phe Gly Arg Leu 1760 1765 1770			5570
GAG TGC AGT GAA GAC AAC CCC TGC GAG GGC CTG AGC AGG CAC GCC ACC Glu Cys Ser Glu Asp Asn Pro Cys Glu Gly Leu Ser Arg His Ala Thr 1775 1780 1785 1790			5618
TTC AGC AAC TTC GGC ATG GCC TTC CTC ACG CTG TTC CGC GTG TCC ACG Phe Ser Asn Phe Gly Met Ala Phe Leu Thr Leu Phe Arg Val Ser Thr 1795 1800 1805			5666
GGG GAC AAC TGG AAC GGG ATC ATG AAG GAC ACG CTG CGC GAG TGC TCC Gly Asp Asn Trp Asn Gly Ile Met Lys Asp Thr Leu Arg Glu Cys Ser 1810 1815 1820			5714
CGT GAG GAC AAG CAC TGC CTG AGC TAC CTG CCG GCC CTG TCG CCC GTC Arg Glu Asp Lys His Cys Leu Ser Tyr Leu Pro Ala Leu Ser Pro Val 1825 1830 1835			5762
TAC TTC GTG ACC TTC GTG CTG GTG GCC CAG TTC GTG CTG GTG AAC GTG Tyr Phe Val Thr Phe Val Leu Val Ala Gln Phe Val Leu Val Asn Val 1840 1845 1850			5810
GTG GTG GCC GTG CTC ATG AAG CAC CTG GAG GAG AGC AAC AAG GAG GCA Val Val Ala Val Leu Met Lys His Leu Glu Glu Ser Asn Lys Glu Ala 1855 1860 1865 1870			5858
CGG GAG GAT GCG GAG CTG GAC GCC GAG ATC GAG CTG GAG ATG GCG CAG Arg Glu Asp Ala Glu Leu Asp Ala Glu Ile Glu Leu Glu Met Ala Gln 1875 1880 1885			5906

GGC CCC GGG AGT GCA CGC CGG GTG GAC GCG GAC AGG CCT CCC TTG CCC Gly Pro Gly Ser Ala Arg Arg Val Asp Ala Asp Arg Pro Pro Leu Pro 1890 1895 1900	5954
CAG GAG AGT CCG GGC GCC AGG GAT GCC CCA AAC CTG GTT GCA CGC AAG Gln Glu Ser Pro Gly Ala Arg Asp Ala Pro Asn Leu Val Ala Arg Lys 1905 1910 1915	6002
GTG TCC GTG TCC AGG ATG CTC TCG CTG CCC AAC GAC AGC TAC ATG TTC Val Ser Val Ser Arg Met Leu Ser Leu Pro Asn Asp Ser Tyr Met Phe 1920 1925 1930	6050
AGG CCC GTG GTG CCT GCC TCG GCG CCC CAC CCC CGC CCG CTG CAG GAG Arg Pro Val Val Pro Ala Ser Ala Pro His Pro Arg Pro Leu Gln Glu 1935 1940 1945 1950	6098
GTG GAG ATG GAG ACC TAT GGG GCC GGC ACC CCC TTG GGC TCC GTT GCC Val Glu Met Glu Thr Tyr Gly Ala Gly Thr Pro Leu Gly Ser Val Ala 1955 1960 1965	6146
TCT GTG CAC TCT CCG CCC GCA GAG TCC TGT GCC TCC CTC CAG ATC CCA Ser Val His Ser Pro Pro Ala Glu Ser Cys Ala Ser Leu Gln Ile Pro 1970 1975 1980	6194
CTG GCT GTG TCG TCC CCA GCC AGG AGC GGC GAG CCC CTC CAC GCC CTG Leu Ala Val Ser Ser Pro Ala Arg Ser Gly Glu Pro Leu His Ala Leu 1985 1990 1995	6242
TCC CCT CGG GGC ACA GCC CGC TCC CCC AGT CTC AGC CGG CTG CTC TGC Ser Pro Arg Gly Thr Ala Arg Ser Pro Ser Leu Ser Arg Leu Leu Cys 2000 2005 2010	6290
AGA CAG GAG GCT GTG CAC ACC GAT TCC TTG GAA GGG AAG ATT GAC AGC Arg Gln Glu Ala Val His Thr Asp Ser Leu Glu Gly Lys Ile Asp Ser 2015 2020 2025 2030	6338
CCT AGG GAC ACC CTG GAT CCT GCA GAG CCT GGT GAG AAA ACC CCG GTG Pro Arg Asp Thr Leu Asp Pro Ala Glu Pro Gly Glu Lys Thr Pro Val 2035 2040 2045	6386
AGG CCG GTG ACC CAG GGG GGC TCC CTG CAG TCC CCA CCA CGC TCC CCA Arg Pro Val Thr Gln Gly Gly Ser Leu Gln Ser Pro Pro Arg Ser Pro 2050 2055 2060	6434
CGG CCC GCC AGC GTC CGC ACT CGT AAG CAT ACC TTC GGA CAG CAC TGC Arg Pro Ala Ser Val Arg Thr Arg Lys His Thr Phe Gly Gln His Cys 2065 2070 2075	6482
GTC TCC AGC CGG CCG GCG GCC CCA GGC GGA GAG GAG GCC GAG GCC TCG Val Ser Ser Arg Pro Ala Ala Pro Gly Gly Glu Ala Glu Ala Ser 2080 2085 2090	6530
GAC CCA GCC GAC GAG GAG GTC AGC CAC ATC ACC AGC TCC GCC TGC CCC Asp Pro Ala Asp Glu Glu Val Ser His Ile Thr Ser Ser Ala Cys Pro 2095 2100 2105 2110	6578
TGG CAG CCC ACA GCC GAG CCC CAT GGC CCC GAA GCC TCT CCG GTG GCC Trp Gln Pro Thr Ala Glu Pro His Gly Pro Glu Ala Ser Pro Val Ala	6626

2115	2120	2125	
GGC GGC GAG CGG GAC CTG CGC AGG CTC TAC AGC GTG GAC GCT CAG GGC Gly Gly Glu Arg Asp Leu Arg Arg Leu Tyr Ser Val Asp Ala Gln Gly 2130 2135 2140			6674
TTC CTG GAC AAG CCG GGC CGG GCA GAC GAG CAG TGG CGG CCC TCG GCG Phe Leu Asp Lys Pro Gly Arg Ala Asp Glu Gln Trp Arg Pro Ser Ala 2145 2150 2155			6722
GAG CTG GGC AGC GGG GAG CCT GGG GAG GCG AAG GCC TGG GGC CCT GAG Glu Leu Gly Ser Gly Glu Pro Gly Glu Ala Lys Ala Trp Gly Pro Glu 2160 2165 2170			6770
GCC GAG CCC GCT CTG GGT GCG CGC AGA AAG AAG AAG ATG AGC CCC CCC Ala Glu Pro Ala Leu Gly Ala Arg Arg Lys Lys Lys Met Ser Pro Pro 2175 2180 2185 2190			6818
TGC ATC TCG GTG GAA CCC CCT GCG GAG GAC GAG GGC TCT GCG CGG CCC Cys Ile Ser Val Glu Pro Pro Ala Glu Asp Glu Gly Ser Ala Arg Pro 2195 2200 2205			6866
TCC GCG GCA GAG GGC GGC AGC ACC ACA CTG AGG CGC AGG ACC CCG TCC Ser Ala Ala Glu Gly Gly Ser Thr Thr Leu Arg Arg Arg Thr Pro Ser 2210 2215 2220			6914
TGT GAG GCC ACG CCT CAC AGG GAA TCC CTG GAG CCC ACA GAG GGC TCA Cys Glu Ala Thr Pro His Arg Glu Ser Leu Glu Pro Thr Glu Gly Ser 2225 2230 2235			6962
GGC GCC GGG GGG GAC CCT GCA GCC AAG GGG GAG CGC TGG GGC CAG GCC Gly Ala Gly Gly Asp Pro Ala Ala Lys Gly Glu Arg Trp Gly Gln Ala 2240 2245 2250			7010
TCC TGC CGG GCT GAG CAC CTG ACC GTC CCC AGC TTT GCC TTT GAG CCG Ser Cys Arg Ala Glu His Leu Thr Val Pro Ser Phe Ala Phe Glu Pro 2255 2260 2265 2270			7058
CTG GAC CTC GGG GTC CCC AGT GGA GAC CCT TTT TTG GAC GGT AGC CAC Leu Asp Leu Gly Val Pro Ser Gly Asp Pro Phe Leu Asp Gly Ser His 2275 2280 2285			7106
AGT GTG ACC CCA GAA TCC AGA GCT TCC TCT TCA GGG GCC ATA GTG CCC Ser Val Thr Pro Glu Ser Arg Ala Ser Ser Ser Gly Ala Ile Val Pro 2290 2295 2300			7154
CTG GAA CCC CCA GAA TCA GAG CCT CCC ATG CCC GTC GGT GAC CCC CCA Leu Glu Pro Pro Glu Ser Glu Pro Pro Met Pro Val Gly Asp Pro Pro 2305 2310 2315			7202
GAG AAG AGG CGG GGG CTG TAC CTC ACA GTC CCC CAG TGT CCT CTG GAG Glu Lys Arg Arg Gly Leu Tyr Leu Thr Val Pro Gln Cys Pro Leu Glu 2320 2325 2330			7250
AAA CCA GGG TCC CCC TCA GCC ACC CCT GCC CCA GGG GGT GGT GCA GAT Lys Pro Gly Ser Pro Ser Ala Thr Pro Ala Pro Gly Gly Gly Ala Asp 2335 2340 2345 2350			7298



GAC CCC GTG TAGCTCGGGG CTTGGTGCCG CCCACGGCTT TGGCCCTGGG GTCTGGGGGG  
7357

Asp Pro Val

CCCGCTGGGG	TGGAGGCCCA	GGCAGAACCC	TGCATGGACC	CTGACTTGGG	TCCCGTCGTG	7417
AGCAGAAAGG	CCCGGGGAGG	ATGACGGCCC	AGGCCCTGGT	TCTCTGCCCC	GCGAAGCAGG	7477
AGTAGCTGCC	GGGCCCCACG	AGCCTCCATC	CGTTCTGGTT	CGGGTTTCTC	CGAGTTTTCG	7537
TACCAGCCGA	GGCTGTGCGG	GCAACTGGGT	CAGCCTCCCG	TCAGGAGAGA	AGCCGCGTCT	7597
GTGGGACGAA	GACCGGGCAC	CCGCCAGAGA	GGGGAAGGTA	CCAGGTTGCG	TCCTTTCAGG	7657
CCCCGCGTTG	TTACAGGACA	CTCGCTGGGG	GCCCTGTGCC	CTTGCCGGCG	GCAGGTTGCA	7717
GCCACGCGGG	CCCAATGTCA	CCTTCACTCA	CAGTCTGAGT	TCTTGTCCGC	CTGTCACGCC	7777
CTCACCACCC	TCCCCTTCCA	GCCACCACCC	TTTCCGTTCC	GCTCGGGCCT	TCCCAGAAGC	7837
GTCTGTGAC	TCTGGGAGAG	GTGACACCTC	ACTAAGGGGC	CGACCCCATG	GAGTAACGCG	7897
C						7898

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1669 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTGCGGCTCC	GAGCGCTGCA	ACATCCTGGA	GGCCTTTGAC	GCCTTCATTT	TCGCCTTTTT	60
TGCGGTGGAG	ATGGTCATCA	AGATGGTGCC	CTTGGGGCTG	TTCGGGCAGA	AGTGTTACCT	120
GGGTGACACG	TGGAACAGGC	TGGATTTCTT	CATCGTCGTG	GCGGGCATGA	TGGAGTACTC	180
GTTGGACGGA	CACAACGTGA	GCCTCTCGGC	TATCAGGACC	GTGCGGGTGC	TGCGGGCCCT	240
CCGCGCCATC	AACCGCGTGC	CTAGCATGCG	GATCCTGGTC	ACTCTGCTGC	TGGATACGCT	300
GCCCATGCTC	GGGAACGTCC	TTCTGCTGTG	CTTCTTCGTC	TTCTTCATTT	TCGGCATCGT	360
TGGCGTCCAG	CTCTGGGCTG	GCCTCCTGCG	GAACCGCTGC	TTCTTGACA	GTGCCTTTGT	420
CAGGAACAAC	AACCTGACCT	TCCTGCGGCC	GTACTACCAG	ACGGAGGAGG	GCGAGGAGAA	480
CCCGTTTCATC	TGCTCCTCAC	GCCGAGACAA	CGGCATGCAG	AAGTGCTCGC	ACATCCCCGG	540
CCGCCGCGAG	CTGCGCATGC	CCTGCACCCCT	GGGCTGGGAG	GCCTACACGC	AGCCGCAGGC	600
CGAGGGGGTG	GGCGCTGCAC	GCAACGCCTG	CATCAACTGG	AACCAGTACT	ACAACGTGTG	660
CCGCTCGGGT	GACTCCAACC	CCCACAACGG	TGCCATCAAC	TTCGACAACA	TCGGCTACGC	720
CTGGATTGCC	ATCTTCCAGG	TGATCACGCT	GGAAGGCTGG	GTGGACATCA	TGTACTACGT	780
CATGGACGCC	CACTCATTCT	ACAACCTTCAT	CTATTTTCATC	CTGCTCATCA	TCGTGGGCTC	840
CTTCTTCATG	ATCAACCTGT	GCCTGGTGGT	GATTGCCACG	CAGTTCTCGG	AGACGAAGCA	900
GCGGGAGAGT	CAGCTGATGC	GGGAGCAGCG	GGCACGCCAC	CTGTCCAACG	ACAGCACGCT	960
GGCCAGCTTC	TCCGAGCCTG	GCAGCTGCTA	CGAAGAGCTG	CCCGTACTGC	ACCCGTGCCC	1020
TGGAGGACCC	GGAGGGTGAG	CTCAGCGGCT	CGGAAAGTGG	AGACTCAGAT	GGCCGTGGCG	1080
TCTATGAATT	CACGCAGGAC	GTCCGGCACG	GTGACCGCTG	GGACCCACAG	CGACCACCCC	1140
GGGCGAGCCA	GGCTGGATGG	GCCGCCTCTG	GGTTACCTTC	AGCGGCAAGC	TGCGCCGCAT	1200
CGTGGACAGC	AAGTACTTCA	GCCGTGGCAT	CATGATGGCC	ATCCTTGTC	ACACGCTGAG	1260
CATGGGCGTG	GAGTACCATG	AGCAGCCCGA	GGAGCTGACT	AATGCTCTGG	AGATCAGCAA	1320
CATCGTGTTC	ACCAGCATGT	TTGCCCTGGA	GATGCTGCTG	AAGCTGCTGG	CCTGCGGCCC	1380
TCTGGGCTAC	ATCCGGAACC	CGTACAACAT	CTTCGACGGC	ATCATCGTGG	TCATCAGCGT	1440
CTGGGAGATC	GTGGGGCAGG	CGGACGGTGG	CTTGTCTGTG	CTGCGCACCT	TCCGGCTGCT	1500
GCGTGTGCTG	AAGCTGGTGC	GCTTTCTGCC	AGCCCTGCGG	CGCCAGCTCG	TGGTGCTGGT	1560

GAAGACCATG GACAACGTGG CTACCTTCTG CACGCTGCTC ATGCTCTTCA TTTTCATCTT 1620  
CAGCATCCTG GGCATGCACC TTTTCGGCTG GCAAGTTCAG CCTGAAGAA 1669

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1413 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACGGGCTCGA	GGCTCGCTCG	CTGCCTCACC	GGTCCCCGGC	CCGCGCCCCG	CGCCCCGCGC	60
CCCCGCGCCC	GGCCTCACCC	GTCCGCTCAG	CGGCCTCCAC	GCCGCGCCGA	GGCCGCGGCC	120
GTCGCTCCG	CCGGGCGAGC	CGGAGCCGGA	GTCGAGCCGC	GGCCGGGAGC	CGGGCGGGCT	180
GGGGACGCGG	GCCGGGGGCG	GAGGCGCTGG	GGGCCGGGGC	CGGGGCGGGG	CGCCGAGCGG	240
GGTCCGCGGT	GACCGCGCCG	CCCGGGCGAT	GCCCGCGGGG	ACGCCGCCGG	CCAGCAGAGC	300
GAGGCATGCG	GATCCTGGTC	ACTCTGCTGC	TGGATACGCT	GCCCATGCTC	GGGAACGTCC	360
TTCTGCTGTG	CTTCTTCGTC	TTCTTCATTT	TCGGCATCGT	TGGCGTCCAG	CTCTGGGCTG	420
GCCTCCTGCG	GAACCGCTGC	TTCTGGACA	GTGCCTTTGT	CAGGAACAAC	AACCTGACCT	480
TCCTGCGGCC	GTACTACCAG	ACGGAGGAGG	GCGAGGAGAA	CCCGTTCATC	TGCTCCTCAC	540
GCCGAGACAA	CGGCATGCAG	AAGTGCTCGC	ACATCCCCGG	CCGCCGCGAG	CTGCGCATGC	600
CCTGCACCCT	GGGCTGGGAG	GCCTACACGC	AGCCGCGAGG	CGAGGGGGTG	GGCGCTGCAC	660
GCAACGCCTG	CATCAACTGG	AACCAGTACT	ACAACGTGTG	CCGCTCGGGT	GACTCCAACC	720
CCCACAACGG	TGCCATCAAC	TTCGACAACG	TGGCTACGC	CTGGATTGCC	ATCTTCCAGG	780
TGATCACGCT	GGAAGGCTGG	GTGGACATCA	TGTACTACGT	CATGGACGCC	CACTCATTCT	840
ACAAC TTCAT	CTATTTTCATC	CTGCTCATCA	TCGTGGGCTC	CTTCTTCATG	ATCAACCTGT	900
GCCTGGTGGT	GATTGCCACG	CAGTTCCTCG	AGACGAAGCA	GCGGGAGAGT	CAGCTGATGC	960
GGGAGCAGCG	GGCAGCCAC	CTGTCCAACG	ACAGCACGCT	GGCCAGCTTC	TCCGAGCCTG	1020
GCAGCTGCTA	CGAAGAGCTG	CTGAAGACTG	GGCCAGGCC	CTGGCCATCT	GTCGGGCTC	1080
AGTGTGCCCT	GCCCCCTGCC	CAGCCCCCCA	GCGGGCACAC	TGACCTGTGA	GCTGAAGAGC	1140
TGCCCCGTA	GCACCCGTGC	CCTGGAGGAC	CCGGAGGGTG	AGCTCAGCGG	CTCGGAAAGT	1200
GGAGACTCAG	ATGGCCGTGG	CGTCTATGAA	TTCACGCAGG	ACGTCCGGCA	CGGTGACCGC	1260
TGGGACCCCA	CGCGACCACC	CCGTGCGACG	GACACACCAG	GCCCAGGCC	AGGCAGCCCC	1320
CAGCGGCGGG	CACAGCAGAG	GGCAGCCCCG	GCGGAGCCAG	GCTGGATGGG	CCGCCTCTGG	1380
GTTACTTCAG	CGGCAAGCTG	CGCGCATCGT	GGA			1413

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7898 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

## (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence  
 (B) LOCATION: 249...7307  
 (D) OTHER INFORMATION:  $\alpha_{1H-1}$

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15

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cgagggccgcc gccgtcgctt ccggccggggcg agccggagacc ggagtcgagc cgcggccgggg 60
agccggggcgg gctggggacg cggggccgggg gcggaggcgc tggggggccgg ggccgggggcc 120
ggggggcggag gcgctggggg ccggggccgg ggccggggcgc cgagcgggggt ccgcggtgac 180
cgcgcgcgcc gggcgatgcc cgcggggacg ccggccggcca gcagagcgag gtgctgccgg 240

ccgccacc atg acc gag ggc gca cgg gcc gcc gac gag gtc cgg gtg ccc 290
      Met Thr Glu Gly Ala Arg Ala Ala Asp Glu Val Arg Val Pro
           1             5             10

ctg ggc gcg ccg ccc cct ggc cct gcg gcg ttg gtg ggg gcg tcc ccg 338
Leu Gly Ala Pro Pro Pro Gly Pro Ala Ala Leu Val Gly Ala Ser Pro
   15             20             25             30

gag agc ccc ggg gcg ccg gga cgc gag gcg gag cgg ggg tcc gag ctc 386
Glu Ser Pro Gly Ala Pro Gly Arg Glu Ala Glu Arg Gly Ser Glu Leu
           35             40             45

ggc gtg tca ccc tcc gag agc ccg gcg gcc gag cgc ggc gcg gag ctg 434
Gly Val Ser Pro Ser Glu Ser Pro Ala Ala Glu Arg Gly Ala Glu Leu
           50             55             60

ggt gcc gac gag gag cag cgc gtc ccg tac ccg gcc ttg gcg gcc acg 482
Gly Ala Asp Glu Glu Gln Arg Val Pro Tyr Pro Ala Leu Ala Ala Thr
           65             70             75

gtc ttc ttc tgc ctc ggt cag acc acg cgg ccg cgc agc tgg tgc ctc 530
Val Phe Phe Cys Leu Gly Gln Thr Thr Arg Pro Arg Ser Trp Cys Leu
           80             85             90

cgg ctg gtc tgc aac cca tgg ttc gag cac gtg agc atg ctg gta atc 578
Arg Leu Val Cys Asn Pro Trp Phe Glu His Val Ser Met Leu Val Ile
           95             100             105

atg ctc aac tgc gtg acc ctg ggc atg ttc cgg ccc tgt gag gac gtt 626
Met Leu Asn Cys Val Thr Leu Gly Met Phe Arg Pro Cys Glu Asp Val
   110             115             120             125

gag tgc ggc tcc gag cgc tgc aac atc ctg gag gcc ttt gag gcc ttc 674
Glu Cys Gly Ser Glu Arg Cys Asn Ile Leu Glu Ala Phe Asp Ala Phe
           130             135             140

att ttc gcc ttt ttt gcg gtg gag atg gtc atc aag atg gtg gcc ttg 722
Ile Phe Ala Phe Phe Ala Val Glu Met Val Ile Lys Met Val Ala Leu
           145             150             155

ggg ctg ttc ggg cag aag tgt tac ctg ggt gac acg tgg aac agg ctg 770
Gly Leu Phe Gly Gln Lys Cys Tyr Leu Gly Asp Thr Trp Asn Arg Leu
   160             165             170

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gat ttc ttc atc gtc gtg gcg ggc atg atg gag tac tcg ttg gac gga Asp Phe Phe Ile Val Val Ala Gly Met Met Glu Tyr Ser Leu Asp Gly 175 180 185 190	818
cac aac gtg agc ctc tcg gct atc agg acc gtg cgg gtg ctg cgg ccc His Asn Val Ser Leu Ser Ala Ile Arg Thr Val Arg Val Leu Arg Pro 195 200 205	866
ctc cgc gcc atc aac cgc gtg cct agc atg cgg atc ctg gtc act ctg Leu Arg Ala Ile Asn Arg Val Pro Ser Met Arg Ile Leu Val Thr Leu 210 215 220	914
ctg ctg gat acg ctg ccc atg ctc ggg aac gtc ctt ctg ctg tgc ttc Leu Leu Asp Thr Leu Pro Met Leu Gly Asn Val Leu Leu Leu Cys Phe 225 230 235	962
ttc gtc ttc ttc att ttc ggc atc gtt ggc gtc cag ctc tgg gct ggc Phe Val Phe Phe Ile Phe Gly Ile Val Gly Val Gln Leu Trp Ala Gly 240 245 250	1010
ctc ctg cgg aac cgc tgc ttc ctg gac agt gcc ttt gtc agg aac aac Leu Leu Arg Asn Arg Cys Phe Leu Asp Ser Ala Phe Val Arg Asn Asn 255 260 265 270	1058
aac ctg acc ttc ctg cgg ccg tac tac cag acg gag gag ggc gag gag Asn Leu Thr Phe Leu Arg Pro Tyr Tyr Gln Thr Glu Glu Gly Glu Glu 275 280 285	1106
aac ccg ttc atc tgc tcc tca cgc cga gac aac ggc atg cag aag tgc Asn Pro Phe Ile Cys Ser Ser Arg Arg Asp Asn Gly Met Gln Lys Cys 290 295 300	1154
tcg cac atc ccc ggc cgc cgc gag ctg cgc atg ccc tgc acc ctg ggc Ser His Ile Pro Gly Arg Arg Glu Leu Arg Met Pro Cys Thr Leu Gly 305 310 315	1202
tgg gag gcc tac acg cag ccg cag gcc gag ggg gtg ggc gct gca cgc Trp Glu Ala Tyr Thr Gln Pro Gln Ala Glu Gly Val Gly Ala Ala Arg 320 325 330	1250
aac gcc tgc atc aac tgg aac cag tac tac aac gtg tgc cgc tcg ggt Asn Ala Cys Ile Asn Trp Asn Gln Tyr Tyr Asn Val Cys Arg Ser Gly 335 340 345 350	1298
gac tcc aac ccc cac aac ggt gcc atc aac ttc gac aac atc ggc tac Asp Ser Asn Pro His Asn Gly Ala Ile Asn Phe Asp Asn Ile Gly Tyr 355 360 365	1346
gcc tgg att gcc atc ttc cag gtg atc acg ctg gaa ggc tgg gtg gac Ala Trp Ile Ala Ile Phe Gln Val Ile Thr Leu Glu Gly Trp Val Asp 370 375 380	1394
atc atg tac tac gtc atg gac gcc cac tca ttc tac aac ttc atc tat Ile Met Tyr Tyr Val Met Asp Ala His Ser Phe Tyr Asn Phe Ile Tyr 385 390 395	1442
ttc atc ctg ctc atc atc gtg ggc tcc ttc ttc atg atc aac ctg tgc	1490

Phe	Ile	Leu	Leu	Ile	Ile	Val	Gly	Ser	Phe	Phe	Met	Ile	Asn	Leu	Cys		
				400					405					410			
ctg	gtg	gtg	att	gcc	acg	cag	ttc	tcg	gag	acg	aag	cag	cgg	gag	agt	1538	
Leu	Val	Val	Ile	Ala	Thr	Gln	Phe	Ser	Glu	Thr	Lys	Gln	Arg	Glu	Ser		
			415					420					425				
cag	ctg	atg	cgg	gag	cag	cgg	gca	cgc	cac	ctg	tcc	aac	gac	agc	acg	1586	
Gln	Leu	Met	Arg	Glu	Gln	Arg	Ala	Arg	His	Leu	Ser	Asn	Asp	Ser	Thr		
			430				435					440					
ctg	gcc	agc	ttc	tcc	gag	cct	ggc	agc	tgc	tac	gaa	gag	ctg	ctg	aag	1634	
Leu	Ala	Ser	Phe	Ser	Glu	Pro	Gly	Ser	Cys	Tyr	Glu	Glu	Leu	Leu	Lys		
	445				450				455						460		
tac	gtg	ggc	cac	ata	ttc	cgc	aag	gtc	aag	cgg	cgc	agc	ttg	cgc	ctc	1682	
Tyr	Val	Gly	His	Ile	Phe	Arg	Lys	Val	Lys	Arg	Arg	Ser	Leu	Arg	Leu		
				465				470						475			
tac	gcc	cgc	tgg	cag	agc	cgc	tgg	cgc	aag	aag	gtg	gac	ccc	agt	gct	1730	
Tyr	Ala	Arg	Trp	Gln	Ser	Arg	Trp	Arg	Lys	Lys	Val	Asp	Pro	Ser	Ala		
			480				485					490					
gtg	caa	ggc	cag	ggt	ccc	ggg	cac	cgc	cag	cgc	cgg	gca	ggc	agg	cac	1778	
Val	Gln	Gly	Gln	Gly	Pro	Gly	His	Arg	Gln	Arg	Arg	Ala	Gly	Arg	His		
	495				500				505						510		
aca	gcc	tcg	gtg	cac	cac	ctg	gtc	tac	cac	cac	cat	cac	cac	cac	cac	1826	
Thr	Ala	Ser	Val	His	His	Leu	Val	Tyr	His	His	His	His	His	His	His		
				515				520						525			
cac	cac	tac	cat	ttc	agc	cat	ggc	agc	ccc	cgc	agg	ccc	ggc	ccc	gag	1874	
His	His	Tyr	His	Phe	Ser	His	Gly	Ser	Pro	Arg	Arg	Pro	Gly	Pro	Glu		
			530					535					540				
cca	ggc	gcc	tgc	gac	acc	agg	ctg	gtc	cga	gct	ggc	gcg	ccc	ccc	tcg	1922	
Pro	Gly	Ala	Cys	Asp	Thr	Arg	Leu	Val	Arg	Ala	Gly	Ala	Pro	Pro	Ser		
			545				550					555					
cca	cct	tcc	cca	ggc	cgc	gga	ccc	ccc	gac	gca	gag	tct	gtg	cac	agc	1970	
Pro	Pro	Ser	Pro	Gly	Arg	Gly	Pro	Pro	Asp	Ala	Glu	Ser	Val	His	Ser		
			560			565					570						
atc	tac	cat	gcc	gac	tgc	cac	ata	gag	ggg	ccg	cag	gag	agg	gcc	cgg	2018	
Ile	Tyr	His	Ala	Asp	Cys	His	Ile	Glu	Gly	Pro	Gln	Glu	Arg	Ala	Arg		
					580				585						590		
gtg	gca	cat	gcc	gca	gcc	act	gcc	gct	gcc	agc	ctc	agg	ctg	gcc	aca	2066	
Val	Ala	His	Ala	Ala	Ala	Thr	Ala	Ala	Ala	Ser	Leu	Arg	Leu	Ala	Thr		
				595				600						605			
ggg	ctg	ggc	acc	atg	aac	tac	ccc	acg	atc	ctg	ccc	tca	ggg	gtg	ggc	2114	
Gly	Leu	Gly	Thr	Met	Asn	Tyr	Pro	Thr	Ile	Leu	Pro	Ser	Gly	Val	Gly		
			610					615					620				
agc	ggc	aaa	ggc	agc	acc	agc	ccc	gga	ccc	aag	ggg	aag	tgg	gcc	ggt	2162	
Ser	Gly	Lys	Gly	Ser	Thr	Ser	Pro	Gly	Pro	Lys	Gly	Lys	Trp	Ala	Gly		
			625				630					635					

gga ccg cca ggc acc ggg ggg cac ggc ccg ttg agc ttg aac agc cct Gly Pro Pro Gly Thr Gly Gly His Gly Pro Leu Ser Leu Asn Ser Pro 640 645 650	2210
gat ccc tac gag aag atc ccg cat gtg gtc ggg gag cat gga ctg ggc Asp Pro Tyr Glu Lys Ile Pro His Val Val Gly Glu His Gly Leu Gly 655 660 665 670	2258
cag gcc cct ggc cat ctg tcg ggc ctc agt gtg ccc tgc ccc ctg ccc Gln Ala Pro Gly His Leu Ser Gly Leu Ser Val Pro Cys Pro Leu Pro 675 680 685	2306
agc ccc cca gcg ggc aca ctg acc tgt gag ctg aag agc tgc ccg tac Ser Pro Pro Ala Gly Thr Leu Thr Cys Glu Leu Lys Ser Cys Pro Tyr 690 695 700	2354
tgc acc cgt gcc ctg gag gac ccg gag ggt gag ctc agc ggc tcg gaa Cys Thr Arg Ala Leu Glu Asp Pro Glu Gly Glu Leu Ser Gly Ser Glu 705 710 715	2402
agt gga gac tca gat ggc cgt ggc gtc tat gaa ttc acg cag gac gtc Ser Gly Asp Ser Asp Gly Arg Gly Val Tyr Glu Phe Thr Gln Asp Val 720 725 730	2450
cgg cac ggt gac cgc tgg gac ccc acg cga cca ccc cgt gcg acg gac Arg His Gly Asp Arg Trp Asp Pro Thr Arg Pro Pro Arg Ala Thr Asp 735 740 745 750	2498
aca cca ggc cca ggc cca ggc agc ccc cag cgg cgg gca cag cag agg Thr Pro Gly Pro Gly Pro Gly Ser Pro Gln Arg Arg Ala Gln Gln Arg 755 760 765	2546
gca gcc ccg ggc gag cca ggc tgg atg ggc cgc ctc tgg gtt acc ttc Ala Ala Pro Gly Glu Pro Gly Trp Met Gly Arg Leu Trp Val Thr Phe 770 775 780	2594
agc ggc aag ctg cgc cgc atc gtg gac agc aag tac ttc agc cgt ggc Ser Gly Lys Leu Arg Arg Ile Val Asp Ser Lys Tyr Phe Ser Arg Gly 785 790 795	2642
atc atg atg gcc atc ctt gtc aac acg ctg agc atg ggc gtg gag tac Ile Met Met Ala Ile Leu Val Asn Thr Leu Ser Met Gly Val Glu Tyr 800 805 810	2690
cat gag cag ccc gag gag ctg act aat gct ctg gag atc agc aac atc His Glu Gln Pro Glu Glu Leu Thr Asn Ala Leu Glu Ile Ser Asn Ile 815 820 825 830	2738
gtg ttc acc agc atg ttt gcc ctg gag atg ctg ctg aag ctg ctg gcc Val Phe Thr Ser Met Phe Ala Leu Glu Met Leu Leu Lys Leu Leu Ala 835 840 845	2786
tgc ggc cct ctg ggc tac atc cgg aac ccg tac aac atc ttc gac ggc Cys Gly Pro Leu Gly Tyr Ile Arg Asn Pro Tyr Asn Ile Phe Asp Gly	2834

850	855	860	
atc atc gtg gtc atc agc gtc tgg gag atc gtg ggg cag gcg gac ggt Ile Ile Val Val Ile Ser Val Trp Glu Ile Val Gly Gln Ala Asp Gly 865 870 875			2882
ggc ttg tct gtg ctg cgc acc ttc cgg ctg ctg cgt gtg ctg aag ctg Gly Leu Ser Val Leu Arg Thr Phe Arg Leu Leu Arg Val Leu Lys Leu 880 885 890			2930
gtg cgc ttt ctg cca gcc ctg cgg cgc cag ctc gtg gtg ctg gtg aag Val Arg Phe Leu Pro Ala Leu Arg Arg Gln Leu Val Val Leu Val Lys 895 900 905 910			2978
acc atg gac aac gtg gct acc ttc tgc acg ctg ctc atg ctc ttc att Thr Met Asp Asn Val Ala Thr Phe Cys Thr Leu Leu Met Leu Phe Ile 915 920 925			3026
ttc atc ttc agc atc ctg ggc atg cac ctt ttc ggc tgc aag ttc agc Phe Ile Phe Ser Ile Leu Gly Met His Leu Phe Gly Cys Lys Phe Ser 930 935 940			3074
ctg aag aca gac acc gga gac acc gtg cct gac agg aag aac ttc gac Leu Lys Thr Asp Thr Gly Asp Thr Val Pro Asp Arg Lys Asn Phe Asp 945 950 955			3122
tcc ctg ctg tgg gcc atc gtc acc gtg ttc cag atc ctg acc cag gag Ser Leu Leu Trp Ala Ile Val Thr Val Phe Gln Ile Leu Thr Gln Glu 960 965 970			3170
gac tgg aac gtg gtc ctg tac aac ggc atg gcc tcc acc tcc tcc tgg Asp Trp Asn Val Val Leu Tyr Asn Gly Met Ala Ser Thr Ser Ser Trp 975 980 985 990			3218
gcc gcc ctc tac ttc gtg gcc ctc atg acc ttc ggc aac tat gtg ctc Ala Ala Leu Tyr Phe Val Ala Leu Met Thr Phe Gly Asn Tyr Val Leu 995 1000 1005			3266
ttc aac ctg ctg gtg gcc atc ctc gtg gag ggc ttc cag gcg gag ggc Phe Asn Leu Leu Val Ala Ile Leu Val Glu Gly Phe Gln Ala Glu Gly 1010 1015 1020			3314
gat gcc aac aga tcc gac acg gag gag gac aag acg tcg gtc cac ttc Asp Ala Asn Arg Ser Asp Thr Asp Glu Asp Lys Thr Ser Val His Phe 1025 1030 1035			3362
gag gag gac ttc cac aag ctc aga gaa ctc cag acc aca gag ctg aag Glu Glu Asp Phe His Lys Leu Arg Glu Leu Gln Thr Thr Glu Leu Lys 1040 1045 1050			3410
atg tgt tcc ctg gcc gtg acc ccc aac ggg cac ctg gag gga cga ggc Met Cys Ser Leu Ala Val Thr Pro Asn Gly His Leu Glu Gly Arg Gly 1055 1060 1065 1070			3458
agc ctg tcc cct ccc ctc atc atg tgc aca gct gcc acg ccc atg cct Ser Leu Ser Pro Pro Leu Ile Met Cys Thr Ala Ala Thr Pro Met Pro			3506

1075										1080					1085					
acc	ccc	aag	agc	tca	cca	ttc	ctg	gat	gca	gcc	ccc	agc	ctc	cca	gac	3554				
Thr	Pro	Lys	Ser	Ser	Pro	Phe	Leu	Asp	Ala	Ala	Pro	Ser	Leu	Pro	Asp					
			1090					1095					1100							
tct	cgg	cgt	ggc	agc	agc	agc	tcc	ggg	gac	ccg	cca	ctg	gga	gac	cag	3602				
Ser	Arg	Arg	Gly	Ser	Ser	Ser	Ser	Gly	Asp	Pro	Pro	Leu	Gly	Asp	Gln					
			1105					1110					1115							
aag	cct	ccg	gcc	agc	ctc	cga	agt	tct	ccc	tgt	gcc	ccc	tgg	ggc	ccc	3650				
Lys	Pro	Pro	Ala	Ser	Leu	Arg	Ser	Ser	Pro	Cys	Ala	Pro	Trp	Gly	Pro					
			1120					1125					1130							
agt	ggc	gcc	tgg	agc	agc	cgg	cgc	tcc	agc	tgg	agc	agc	ctg	ggc	cgt	3698				
Ser	Gly	Ala	Trp	Ser	Ser	Arg	Arg	Ser	Ser	Trp	Ser	Ser	Leu	Gly	Arg					
			1135				1140						1145		1150					
gcc	ccc	agc	ctc	aag	cgc	cgc	ggc	cag	tgt	ggg	gaa	cgt	gag	tcc	ctg	3746				
Ala	Pro	Ser	Leu	Lys	Arg	Arg	Gly	Gln	Cys	Gly	Glu	Arg	Glu	Ser	Leu					
				1155					1160						1165					
ctg	tct	ggc	gag	ggc	aag	ggc	agc	acc	gac	gac	gaa	gct	gag	gac	ggc	3794				
Leu	Ser	Gly	Glu	Gly	Lys	Gly	Ser	Thr	Asp	Asp	Glu	Ala	Glu	Asp	Gly					
			1170					1175						1180						
agg	gcc	gcg	ccc	ggg	ccc	cgt	gcc	acc	cca	ctg	cgg	cgg	gcc	gag	tcc	3842				
Arg	Ala	Ala	Pro	Gly	Pro	Arg	Ala	Thr	Pro	Leu	Arg	Arg	Ala	Glu	Ser					
			1185					1190						1195						
ctg	gac	cca	cgg	ccc	ctg	cgg	ccg	gcc	gcc	ctc	ccg	cct	acc	aag	tgc	3890				
Leu	Asp	Pro	Arg	Pro	Leu	Arg	Pro	Ala	Ala	Leu	Pro	Pro	Thr	Lys	Cys					
			1200				1205						1210							
cgc	gat	cgc	gac	ggg	cag	gtg	gtg	gcc	ctg	ccc	agc	gac	ttc	ttc	ctg	3938				
Arg	Asp	Arg	Asp	Gly	Gln	Val	Val	Ala	Leu	Pro	Ser	Asp	Phe	Phe	Leu					
			1215				1220			1225					1230					
cgc	atc	gac	agc	cac	cgt	gag	gat	gca	gcc	gag	ctt	gac	gac	gac	tgc	3986				
Arg	Ile	Asp	Ser	His	Arg	Glu	Asp	Ala	Ala	Glu	Leu	Asp	Asp	Asp	Ser					
				1235				1240						1245						
gag	gac	agc	tgc	tgc	ctc	cgc	ctg	cat	aaa	gtg	ctg	gag	ccc	tac	aag	4034				
Glu	Asp	Ser	Cys	Cys	Leu	Arg	Leu	His	Lys	Val	Leu	Glu	Pro	Tyr	Lys					
			1250					1255						1260						
ccc	cag	tgg	tgc	cgg	agc	cgc	gag	gcc	tgg	gcc	ctc	tac	ctc	ttc	tcc	4082				
Pro	Gln	Trp	Cys	Arg	Ser	Arg	Glu	Ala	Trp	Ala	Leu	Tyr	Leu	Phe	Ser					
			1265					1270					1275							
cca	cag	aac	cgg	ttc	cgc	gtc	tcc	tgc	cag	aag	gtc	atc	aca	cac	aag	4130				
Pro	Gln	Asn	Arg	Phe	Arg	Val	Ser	Cys	Gln	Lys	Val	Ile	Thr	His	Lys					
			1280				1285						1290							
atg	ttt	gat	cac	gtg	gtc	ctc	gtc	ttc	atc	ttc	ctc	aac	tgc	gtc	acc	4178				
Met	Phe	Asp	His	Val	Val	Leu	Val	Phe	Ile	Phe	Leu	Asn	Cys	Val	Thr					
				1295		1300			1305					1310						



atc gcc ctg gag agg cct gac att gac ccc ggc agc acc gag cgg gtc Ile Ala Leu Glu Arg Pro Asp Ile Asp Pro Gly Ser Thr Glu Arg Val 1315 1320 1325	4226
ttc ctc agc gtc tcc aat tac atc ttc acg gcc atc ttc gtg gcg gag Phe Leu Ser Val Ser Asn Tyr Ile Phe Thr Ala Ile Phe Val Ala Glu 1330 1335 1340	4274
atg atg gtg aag gtg gtg gcc ctg ggg ctg ctg tcc ggc gag cac gcc Met Met Val Lys Val Val Ala Leu Gly Leu Leu Ser Gly Glu His Ala 1345 1350 1355	4322
tac ctg cag agc agc tgg aac ctg ctg gat ggg ctg ctg gtg ctg gtg Tyr Leu Gln Ser Ser Trp Asn Leu Leu Asp Gly Leu Leu Val Leu Val 1360 1365 1370	4370
tcc ctg gtg gac att gtc gtg gcc atg gcc tgc gct ggt ggc gcc aag Ser Leu Val Asp Ile Val Val Ala Met Ala Ser Ala Gly Gly Ala Lys 1375 1380 1385 1390	4418
atc ctg ggt gtt ctg cgc gtg ctg cgt ctg ctg cgg acc ctg cgg cct Ile Leu Gly Val Leu Arg Val Leu Arg Leu Leu Arg Thr Leu Arg Pro 1395 1400 1405	4466
cta agg gtc atc agc cgg gcc ccg ggc ctc aag ctg gtg gtg gag acg Leu Arg Val Ile Ser Arg Ala Pro Gly Leu Lys Leu Val Val Glu Thr 1410 1415 1420	4514
ctg ata tgc tgc ctc agg ccc att ggg aac atc gtc ctc atc tgc tgc Leu Ile Ser Ser Leu Arg Pro Ile Gly Asn Ile Val Leu Ile Cys Cys 1425 1430 1435	4562
gcc ttc ttc atc att ttt ggc atc ttg ggt gtg cag ctc ttc aaa ggg Ala Phe Phe Ile Ile Phe Gly Ile Leu Gly Val Gln Leu Phe Lys Gly 1440 1445 1450	4610
aag ttc tac tac tgc gag ggc ccc gac acc agg aac atc tcc acc aag Lys Phe Tyr Tyr Cys Glu Gly Pro Asp Thr Arg Asn Ile Ser Thr Lys 1455 1460 1465 1470	4658
gca cag tgc cgg gcc gcc cac tac cgc tgg gtg cga cgc aag tac aac Ala Gln Cys Arg Ala Ala His Tyr Arg Trp Val Arg Arg Lys Tyr Asn 1475 1480 1485	4706
ttc gac aac ctg ggc cag gcc ctg atg tgc ctg ttc gtg ctg tca tcc Phe Asp Asn Leu Gly Gln Ala Leu Met Ser Leu Phe Val Leu Ser Ser 1490 1495 1500	4754
aag gat gga tgg gtg aac atc atg tac gac ggg ctg gat gcc gtg ggt Lys Asp Gly Trp Val Asn Ile Met Tyr Asp Gly Leu Asp Ala Val Gly 1505 1510 1515	4802
gtc gac cag cag cct gtg cag aac cac aac ccc tgg atg ctg ctg tac Val Asp Gln Gln Pro Val Gln Asn His Asn Pro Trp Met Leu Leu Tyr 1520 1525 1530	4850

ttc atc tcc ttc ctg ctc atc gtc agc ttc ttc gtg ctc aac atg ttc	4898
Phe Ile Ser Phe Leu Leu Ile Val Ser Phe Phe Val Leu Asn Met Phe	
1535                      1540                      1545                      1550	
gtg ggc gtc gtg gtc gag aac ttc cac aag tgc cgg cag cac cag gag	4946
Val Gly Val Val Val Glu Asn Phe His Lys Cys Arg Gln His Gln Glu	
1555                      1560                      1565	
gcg gag gag gcg cgg cgg cga gag gag aag cgg ctg cgg cgc cta gag	4994
Ala Glu Glu Ala Arg Arg Arg Glu Glu Lys Arg Leu Arg Arg Leu Glu	
1570                      1575                      1580	
agg agg cgc agg agc act ttc ccc agc cca gag gcc cag cgc cgg ccc	5042
Arg Arg Arg Arg Ser Thr Phe Pro Ser Pro Glu Ala Gln Arg Arg Pro	
1585                      1590                      1595	
tac tat gcc gac tac tcg ccc acg cgc cgc tcc att cac tcg ctg tgc	5090
Tyr Tyr Ala Asp Tyr Ser Pro Thr Arg Arg Ser Ile His Ser Leu Cys	
1600                      1605                      1610	
acc agc cac tat ctc gac ctc ttc atc acc ttc atc atc tgt gtc aac	5138
Thr Ser His Tyr Leu Asp Leu Phe Ile Thr Phe Ile Ile Cys Val Asn	
1615                      1620                      1625                      1630	
gtc atc acc atg tcc atg gag cac tat aac caa ccc aag tcg ctg gac	5186
Val Ile Thr Met Ser Met Glu His Tyr Asn Gln Pro Lys Ser Leu Asp	
1635                      1640                      1645	
gag gcc ctc aag tac tgc aac tac gtc ttc acc atc gtg ttt gtc ttc	5234
Glu Ala Leu Lys Tyr Cys Asn Tyr Val Phe Thr Ile Val Phe Val Phe	
1650                      1655                      1660	
gag gct gca ctg aag ctg gta gca ttt ggg ttc cgt cgg ttc ttc aag	5282
Glu Ala Ala Leu Lys Leu Val Ala Phe Gly Phe Arg Arg Phe Phe Lys	
1665                      1670                      1675	
gac agg tgg aac cag ctg gac ctg gcc atc gtg ctg ctg tca ctc atg	5330
Asp Arg Trp Asn Gln Leu Asp Leu Ala Ile Val Leu Leu Ser Leu Met	
1680                      1685                      1690	
ggc atc acg ctg gag gag ata gag atg agc gcc gcg ctg ccc atc aac	5378
Gly Ile Thr Leu Glu Glu Ile Glu Met Ser Ala Ala Leu Pro Ile Asn	
1695                      1700                      1705                      1710	
ccc acc atc atc cgc atc atg cgc gtg ctt cgc att gcc cgt gtg ctg	5426
Pro Thr Ile Ile Arg Ile Met Arg Val Leu Arg Ile Ala Arg Val Leu	
1715                      1720                      1725	
aag ctg ctg aag atg gct acg ggc atg cgc gcc ctg ctg gac act gtg	5474
Lys Leu Leu Lys Met Ala Thr Gly Met Arg Ala Leu Leu Asp Thr Val	
1730                      1735                      1740	
gtg caa gct ctc ccc cag gtg ggg aac ctg ggc ctt ctt ttc atg ctc	5522
Val Gln Ala Leu Pro Gln Val Gly Asn Leu Gly Leu Leu Phe Met Leu	
1745                      1750                      1755	

ctg ttt ttt atc tat gct gcg ctg gga gtg gag ctg ttc ggg agg ctg 5570  
 Leu Phe Phe Ile Tyr Ala Ala Leu Gly Val Glu Leu Phe Gly Arg Leu  
 1760 1765 1770

gag tgc agt gaa gac aac ccc tgc gag ggc ctg agc agg cac gcc acc 5618  
 Glu Cys Ser Glu Asp Asn Pro Cys Glu Gly Leu Ser Arg His Ala Thr  
 1775 1780 1785 1790

ttc agc aac ttc ggc atg gcc ttc ctc acg ctg ttc cgc gtg tcc acg 5666  
 Phe Ser Asn Phe Gly Met Ala Phe Leu Thr Leu Phe Arg Val Ser Thr  
 1795 1800 1805

ggg gac aac tgg aac ggg atc atg aag gac acg ctg cgc gag tgc tcc 5714  
 Gly Asp Asn Trp Asn Gly Ile Met Lys Asp Thr Leu Arg Glu Cys Ser  
 1810 1815 1820

cgt gag gac ag cac tgc ctg agc tac ctg ccg gcc ctg tcg ccc gtc 5762  
 Arg Glu Asp Lys His Cys Leu Ser Tyr Leu Pro Ala Leu Ser Pro Val  
 1825 1830 1835

tac ttc gtg acc ttc gtg ctg gtg gcc cag ttc gtg ctg gtg aac gtg 5810  
 Tyr Phe Val Thr Phe Val Leu Val Ala Gln Phe Val Leu Val Asn Val  
 1840 1845 1850

gtg gtg gcc gtg ctc atg aag cac ctg gag gag agc aac aag gag gca 5858  
 Val Val Ala Val Leu Met Lys His Leu Glu Glu Ser Asn Lys Glu Ala  
 1855 1860 1865 1870

cgg gag gat gcg gag ctg gac gcc gag atc gag ctg gag atg gcg cag 5906  
 Arg Glu Asp Ala Glu Leu Asp Ala Glu Ile Glu Leu Glu Met Ala Gln  
 1875 1880 1885

ggc ccc ggg agt gca cgc cgg gtg gac gcg gac agg cct ccc ttg ccc 5954  
 Gly Pro Gly Ser Ala Arg Arg Val Asp Ala Asp Arg Pro Pro Leu Pro  
 1890 1895 1900

cag gag agt ccg ggc gcc agg gat gcc cca aac ctg gtt gca cgc aag 6002  
 Gln Glu Ser Pro Gly Ala Arg Asp Ala Pro Asn Leu Val Ala Arg Lys  
 1905 1910 1915

gtg tcc gtg tcc agg atg ctc tcg ctg ccc aac gac agc tac atg ttc 6050  
 Val Ser Val Ser Arg Met Leu Ser Leu Pro Asn Asp Ser Tyr Met Phe  
 1920 1925 1930

agg ccc gtg gtg cct gcc tcg gcg ccc cac ccc cgc ccg ctg cag gag 6098  
 Arg Pro Val Val Pro Ala Ser Ala Pro His Pro Arg Pro Leu Gln Glu  
 1935 1940 1945 1950

gtg gag atg gag acc tat ggg gcc ggc acc ccc ttg ggc tcc gtt gcc 6146  
 Val Glu Met Glu Thr Tyr Gly Ala Gly Thr Pro Leu Gly Ser Val Ala  
 1955 1960 1965

tct gtg cac tct ccg ccc gca gag tcc tgt gcc tcc ctc cag atc cca 6194  
 Ser Val His Ser Pro Pro Ala Glu Ser Cys Ala Ser Leu Gln Ile Pro  
 1970 1975 1980

ctg gct gtg tcg tcc cca gcc agg agc ggc gag ccc ctc cac gcc ctg 6242

Leu Ala Val Ser Ser Pro Ala Arg Ser Gly Glu Pro Leu His Ala Leu	
1985 1990 1995	
tcc cct cgg ggc aca gcc cgc tcc ccc agt ctc agc cgg ctg ctc tgc	6290
Ser Pro Arg Gly Thr Ala Arg Ser Pro Ser Leu Ser Arg Leu Leu Cys	
2000 2005 2010	
aga cag gag gct gtg cac acc gat tcc ttg gaa ggg aag att gac agc	6338
Arg Gln Glu Ala Val His Thr Asp Ser Leu Glu Gly Lys Ile Asp Ser	
2015 2020 2025 2030	
cct agg gac acc ctg gat cct gca gag cct ggt gag aaa acc ccg gtg	6386
Pro Arg Asp Thr Leu Asp Pro Ala Glu Pro Gly Glu Lys Thr Pro Val	
2035 2040 2045	
agg ccg gtg acc cag ggg ggc tcc ctg cag tcc cca cca cgc tcc cca	6434
Arg Pro Val Thr Gln Gly Gly Ser Leu Gln Ser Pro Pro Arg Ser Pro	
2050 2055 2060	
cgg ccc gcc agc gtc cgc act cgt aag cat acc ttc gga cag cac tgc	6482
Arg Pro Ala Ser Val Arg Thr Arg Lys His Thr Phe Gly Gln His Cys	
2065 2070 2075	
gtc tcc agc cgg ccg gcg gcc cca ggc gga gag gag gcc gag gcc tcg	6530
Val Ser Ser Arg Pro Ala Ala Pro Gly Gly Glu Glu Ala Glu Ala Ser	
2080 2085 2090	
gac cca gcc gac gag gag gtc agc cac atc acc agc tcc gcc tgc ccc	6578
Asp Pro Ala Asp Glu Glu Val Ser His Ile Thr Ser Ser Ala Cys Pro	
2095 2100 2105 2110	
tgg cag ccc aca gcc gag ccc cat ggc ccc gaa gcc tct ccg gtg gcc	6626
Trp Gln Pro Thr Ala Glu Pro His Gly Pro Glu Ala Ser Pro Val Ala	
2115 2120 2125	
ggc ggc gag cgg gac ctg cgc agg ctc tac agc gtg gac gct cag ggc	6674
Gly Gly Glu Arg Asp Leu Arg Arg Leu Tyr Ser Val Asp Ala Gln Gly	
2130 2135 2140	
ttc ctg gac aag ccg ggc cgg gca gac gag cag tgg cgg ccc tcg gcg	6722
Phe Leu Asp Lys Pro Gly Arg Ala Asp Glu Gln Trp Arg Pro Ser Ala	
2145 2150 2155	
gag ctg ggc agc ggg gag cct ggg gag gcg aag gcc tgg ggc cct gag	6770
Glu Leu Gly Ser Gly Glu Pro Gly Glu Ala Lys Ala Trp Gly Pro Glu	
2160 2165 2170	
gcc gag ccc gct ctg ggt gcg cgc aga aag aag aag atg agc ccc ccc	6818
Ala Glu Pro Ala Leu Gly Ala Arg Arg Lys Lys Lys Met Ser Pro Pro	
2175 2180 2185 2190	
tgc atc tcg gtg gaa ccc cct gcg gag gac gag ggc tct gcg cgg ccc	6866
Cys Ile Ser Val Glu Pro Pro Ala Glu Asp Glu Gly Ser Ala Arg Pro	
2195 2200 2205	
tcc gcg gca gag ggc ggc agc acc aca ctg agg cgc agg acc ccg tcc	6914
Ser Ala Ala Glu Gly Gly Ser Thr Thr Leu Arg Arg Arg Thr Pro Ser	
2210 2215 2220	

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tgt gag gcc acg cct cac agg gac tcc ctg gag ccc aca gag ggc tca 6962
Cys Glu Ala Thr Pro His Arg Asp Ser Leu Glu Pro Thr Glu Gly Ser
      2225                      2230                      2235

ggc gcc ggg ggg gac cct gca gcc aag ggg gag cgc tgg ggc cag gcc 7010
Gly Ala Gly Gly Asp Pro Ala Ala Lys Gly Glu Arg Trp Gly Gln Ala
      2240                      2245                      2250

tcc tgc cgg gct gag cac ctg acc gtc ccc agc ttt gcc ttt gag ccg 7058
Ser Cys Arg Ala Glu His Leu Thr Val Pro Ser Phe Ala Phe Glu Pro
      2255                      2260                      2265                      2270

ctg gac ctc ggg gtc ccc agt gga gac cct ttc ttg gac ggt agc cac 7106
Leu Asp Leu Gly Val Pro Ser Gly Asp Pro Phe Leu Asp Gly Ser His
      2275                      2280                      2285

agt gtg acc cca gaa tcc aga gct tcc tct tca ggg gcc ata gtg ccc 7154
Ser Val Thr Pro Glu Ser Arg Ala Ser Ser Ser Gly Ala Ile Val Pro
      2290                      2295                      2300

ctg gaa ccc cca gaa tca gag cct ccc atg ccc gtc ggt gac ccc cca 7202
Leu Glu Pro Pro Glu Ser Glu Pro Pro Met Pro Val Gly Asp Pro Pro
      2305                      2310                      2315

gag aag agg cgg ggg ctg tac ctc aca gtc ccc cag tgt cct ctg gag 7250
Glu Lys Arg Arg Gly Leu Tyr Leu Thr Val Pro Gln Cys Pro Leu Glu
      2320                      2325                      2330

aaa cca ggg tcc ccc tca gcc acc cct gcc cca ggg ggt ggt gca gat 7298
Lys Pro Gly Ser Pro Ser Ala Thr Pro Ala Pro Gly Gly Gly Ala Asp
      2335                      2340                      2345                      2350

gac ccc gtg tag ctg ggg ggt ggt gcc gcc acc ggc ggt ggt gca gat 7350
Asp Pro Val
      2355                      2360                      2365                      2370

tggggggcccc gctgggggtgg agggccaggc agaaccctgc atggaccctg acttgggtcc 7410
cgctcgtgagc agaaaggccc ggggaggatg acggccagg ccctggttct ctgcccagcg 7470
aagcaggagt agctgccggg ccccacgagc ctccatccgt tctggttcgg gtttctccga 7530
gttttgctac cagccgaggc tgtgcgggca actgggtcag cctcccgta ggagagaagc 7590
cgcgctctgtg ggacgaagac cgggcaccc cagagagagg gaaggtacca ggttgcgctcc 7650
tttcaggccc cgcgttggtta caggacactc gctggggggc ctgtgccctt gccggcgcca 7710
ggttgcagcc acccgggccc aatgtcacct tcaactcacag tctgagttct tgtccgcctg 7770
tcacgccctc accaccctcc ccttccagcc accaccctt ccgttccgct cgggccttcc 7830
cagaagcgtc ctgtgactct gggagaggtg acacctcact aaggggccga ccccatggag 7890
taacgcgc
      7895

```

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 6941 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 249... 6353

(D) OTHER INFORMATION:  $\alpha_{1H-2}$ 

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16

```

cgaggccgcc gccgtgcct ccgccgggag agccggagcc ggagtcgagc cgcggccggg 60
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gggggaggag gcgctggggg ccggggccgg ggccggggcgc cgagcggggg ccgcggtgac 180
cgcgccgccc gggcgatgcc cgcgggggacg ccgccggcca gcagagcgag gtgctgccg 240

ccgccacc atg acc gag ggc gca cgg gcc gcc gac gag gtc cgg gtg ccc 290
      Met Thr Glu Gly Ala Arg Ala Ala Asp Glu Val Arg Val Pro
        1             5             10

ctg ggc gcg ccg ccc cct ggc cct gcg gcg ttg gtg ggg gcg tcc ccg 338
Leu Gly Ala Pro Pro Gly Pro Ala Ala Leu Val Gly Ala Ser Pro
  15             20             25             30

gag agc ccc ggg gcg ccg gga cgc gag gcg gag cgg ggg tcc gag ctc 386
Glu Ser Pro Gly Ala Pro Gly Arg Glu Ala Glu Arg Gly Ser Glu Leu
          35             40             45

ggc gtg tca ccc tcc gag agc ccg gcg gcc gag cgc ggc gcg gag ctg 434
Gly Val Ser Pro Ser Glu Ser Pro Ala Ala Glu Arg Gly Ala Glu Leu
          50             55             60

ggg gcc gac gag gag cag cgc gtc ccg tac ccg gcc ttg gcg gcc acg 482
Gly Ala Asp Glu Glu Gln Arg Val Pro Tyr Pro Ala Leu Ala Ala Thr
        65             70             75

gtc ttc ttc tgc ctc ggt cag acc acg cgg ccg cgc agc tgg tgc ctc 530
Val Phe Phe Cys Leu Gly Gln Thr Thr Arg Pro Arg Ser Trp Cys Leu
      80             85             90

cgg ctg gtc tgc aac cca tgg ttc gag cac gtg agc atg ctg gta atc 578
Arg Leu Val Cys Asn Pro Trp Phe Glu His Val Ser Met Leu Val Ile
      95             100             105             110

atg ctc aac tgc gtg acc ctg ggc atg ttc cgg ccc tgt gag gac gtt 626
Met Leu Asn Cys Val Thr Leu Gly Met Phe Arg Pro Cys Glu Asp Val
          115             120             125

gag tgc ggc tcc gag cgc tgc aac atc ctg gag gcc ttt gac gcc ttc 674
Glu Cys Gly Ser Glu Arg Cys Asn Ile Leu Glu Ala Phe Asp Ala Phe
          130             135             140

att ttc gcc ttt ttt gcg gtg gag atg gtc atc aag atg gtg gcc ttg 722
Ile Phe Ala Phe Phe Ala Val Glu Met Val Ile Lys Met Val Ala Leu
          145             150             155

ggg ctg ttc ggg cag aag tgt tac ctg ggt gac acg tgg aac agg ctg 770

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Gly	Leu	Phe	Gly	Gln	Lys	Cys	Tyr	Leu	Gly	Asp	Thr	Trp	Asn	Arg	Leu		
160						165					170						
gat	ttc	ttc	atc	gtc	gtg	gcg	ggc	atg	atg	gag	tac	tcg	ttg	gac	gga	818	
Asp	Phe	Phe	Ile	Val	Val	Ala	Gly	Met	Met	Glu	Tyr	Ser	Leu	Asp	Gly		
175					180					185					190		
cac	aac	gtg	agc	ctc	tcg	gct	atc	agg	acc	gtg	cgg	gtg	ctg	cgg	ccc	866	
His	Asn	Val	Ser	Leu	Ser	Ala	Ile	Arg	Thr	Val	Arg	Val	Leu	Arg	Pro		
				195					200					205			
ctc	cgc	gcc	atc	aac	cgc	gtg	cct	agc	atg	cgg	atc	ctg	gtc	act	ctg	914	
Leu	Arg	Ala	Ile	Asn	Arg	Val	Pro	Ser	Met	Arg	Ile	Leu	Val	Thr	Leu		
			210					215					220				
ctg	ctg	gat	acg	ctg	ccc	atg	ctc	ggg	aac	gtc	ctt	ctg	ctg	tgc	ttc	962	
Leu	Leu	Asp	Thr	Leu	Pro	Met	Leu	Gly	Asn	Val	Leu	Leu	Leu	Cys	Phe		
		225					230					235					
ttc	gtc	ttc	ttc	att	ttc	ggc	atc	gtt	ggc	gtc	cag	ctc	tgg	gct	ggc	1010	
Phe	Val	Phe	Phe	Ile	Phe	Gly	Ile	Val	Gly	Val	Gln	Leu	Trp	Ala	Gly		
	240					245					250						
ctc	ctg	cgg	aac	cgc	tgc	ttc	ctg	gac	agt	gcc	ttt	gtc	agg	aac	aac	1058	
Leu	Leu	Arg	Asn	Arg	Cys	Phe	Leu	Asp	Ser	Ala	Phe	Val	Arg	Asn	Asn		
255					260					265					270		
aac	ctg	acc	ttc	ctg	cgg	ccg	tac	tac	cag	acg	gag	gag	ggc	gag	gag	1106	
Asn	Leu	Thr	Phe	Leu	Arg	Pro	Tyr	Tyr	Gln	Thr	Glu	Glu	Gly	Glu	Glu		
				275					280					285			
aac	ccg	ttc	atc	tgc	tcc	tca	cgc	cga	gac	aac	ggc	atg	cag	aag	tgc	1154	
Asn	Pro	Phe	Ile	Cys	Ser	Ser	Arg	Arg	Asp	Asn	Gly	Met	Gln	Lys	Cys		
			290					295					300				
tcg	cac	atc	ccc	ggc	cgc	cgc	gag	ctg	cgc	atg	ccc	tgc	acc	ctg	ggc	1202	
Ser	His	Ile	Pro	Gly	Arg	Arg	Glu	Leu	Arg	Met	Pro	Cys	Thr	Leu	Gly		
		305					310					315					
tgg	gag	gcc	tac	acg	cag	ccg	cag	gcc	gag	ggg	gtg	ggc	gct	gca	cgc	1250	
Trp	Glu	Ala	Tyr	Thr	Gln	Pro	Gln	Ala	Glu	Gly	Val	Gly	Ala	Ala	Arg		
	320					325					330						
aac	gcc	tgc	atc	aac	tgg	aac	cag	tac	tac	aac	gtg	tgc	cgc	tcg	ggc	1298	
Asn	Ala	Cys	Ile	Asn	Trp	Asn	Gln	Tyr	Tyr	Asn	Val	Cys	Arg	Ser	Gly		
335					340					345					350		
gac	tcc	aac	ccc	cac	aac	ggc	gcc	atc	aac	ttc	gac	aac	atc	ggc	tac	1346	
Asp	Ser	Asn	Pro	His	Asn	Gly	Ala	Ile	Asn	Phe	Asp	Asn	Ile	Gly	Tyr		
				355					360					365			
gcc	tgg	att	gcc	atc	ttc	cag	gtg	atc	acg	ctg	gaa	ggc	tgg	gtg	gac	1394	
Ala	Trp	Ile	Ala	Ile	Phe	Gln	Val	Ile	Thr	Leu	Glu	Gly	Trp	Val	Asp		
			370					375					380				
atc	atg	tac	tac	gtc	atg	gac	gcc	cac	tca	ttc	tac	aac	ttc	atc	tat	1442	
Ile	Met	Tyr	Tyr	Val	Met	Asp	Ala	His	Ser	Phe	Tyr	Asn	Phe	Ile	Tyr		
		385					390					395					

ttc atc ctg ctc atc atc gtg ggc tcc ttc ttc atg atc aac ctg tgc	1490
Phe Ile Leu Leu Ile Ile Val Gly Ser Phe Phe Met Ile Asn Leu Cys	
400 405 410	
ctg gtg gtg att gcc acg cag ttc tcg gag acg aag cag cgg gag agt	1538
Leu Val Val Ile Ala Thr Gln Phe Ser Glu Thr Lys Gln Arg Glu Ser	
415 420 425 430	
cag ctg atg cgg gag cag cgg gca cgc cac ctg tcc aac gac agc acg	1586
Gln Leu Met Arg Glu Gln Arg Ala Arg His Leu Ser Asn Asp Ser Thr	
435 440 445	
ctg gcc agc ttc tcc gag cct ggc agc tgc tac gaa gag ctg ctg aag	1634
Leu Ala Ser Phe Ser Glu Pro Gly Ser Cys Tyr Glu Glu Leu Leu Lys	
450 455 460	
tac gtg ggc cac ata ttc cgc atc gtg gac agc aag tac ttc agc cgt	1682
Tyr Val Gly His Ile Phe Arg Ile Val Asp Ser Lys Tyr Phe Ser Arg	
465 470 475	
ggc atc atg atg gcc atc ctt gtc aac acg ctg agc atg ggc gtg gag	1730
Gly Ile Met Met Ala Ile Leu Val Asn Thr Leu Ser Met Gly Val Glu	
480 485 490	
tac cat gag cag ccc gag gag ctg act aat gct ctg gag atc agc aac	1778
Tyr His Glu Gln Pro Glu Glu Leu Thr Asn Ala Leu Glu Ile Ser Asn	
495 500 505 510	
atc gtg ttc acc agc atg ttt gcc ctg gag atg ctg ctg aag ctg ctg	1826
Ile Val Phe Thr Ser Met Phe Ala Leu Glu Met Leu Leu Lys Leu Leu	
515 520 525	
gcc tgc ggc cct ctg ggc tac atc cgg aac ccg tac aac atc ttc gac	1874
Ala Cys Gly Pro Leu Gly Tyr Ile Arg Asn Pro Tyr Asn Ile Phe Asp	
530 535 540	
ggc atc atc gtg gtc atc agc gtc tgg gag atc gtg ggg cag gcg gac	1922
Gly Ile Ile Val Val Ile Ser Val Trp Glu Ile Val Gly Gln Ala Asp	
545 550 555	
ggg ggc ttg tct gtg ctg cgc acc ttc cgg ctg ctg cgt gtg ctg aag	1970
Gly Gly Leu Ser Val Leu Arg Thr Phe Arg Leu Leu Arg Val Leu Lys	
560 565 570	
ctg gtg cgc ttt ctg cca gcc ctg cgg cgc cag ctc gtg gtg ctg gtg	2018
Leu Val Arg Phe Leu Pro Ala Leu Arg Arg Gln Leu Val Val Leu Val	
575 580 585 590	
aag acc atg gac aac gtg gct acc ttc tgc acg ctg ctc atg ctc ttc	2066
Lys Thr Met Asp Asn Val Ala Thr Phe Cys Thr Leu Leu Met Leu Phe	
595 600 605	
att ttc atc ttc agc atc ctg ggc atg cac ctt ttc ggc tgc aag ttc	2114
Ile Phe Ile Phe Ser Ile Leu Gly Met His Leu Phe Gly Cys Lys Phe	
610 615 620	
agc ctg aag aca gac acc gga gac acc gtg cct gac agg aag aac ttc	2162



Ser	Leu	Lys	Thr	Asp	Thr	Gly	Asp	Thr	Val	Pro	Asp	Arg	Lys	Asn	Phe		
		625					630					635					
gac	tcc	ctg	ctg	tgg	gcc	atc	gtc	acc	gtg	ttc	cag	atc	ctg	acc	cag	2210	
Asp	Ser	Leu	Leu	Trp	Ala	Ile	Val	Thr	Val	Phe	Gln	Ile	Leu	Thr	Gln		
	640					645					650						
gag	gac	tgg	aac	gtg	gtc	ctg	tac	aac	ggc	atg	gcc	tcc	acc	tcc	tcc	2258	
Glu	Asp	Trp	Asn	Val	Val	Leu	Tyr	Asn	Gly	Met	Ala	Ser	Thr	Ser	Ser		
	655				660					665					670		
tgg	gcc	gcc	ctc	tac	ttc	gtg	gcc	ctc	atg	acc	ttc	ggc	aac	tat	gtg	2306	
Trp	Ala	Ala	Leu	Tyr	Phe	Val	Ala	Leu	Met	Thr	Phe	Gly	Asn	Tyr	Val		
				675					680					685			
ctc	ttc	aac	ctg	ctg	gtg	gcc	atc	ctc	gtg	gag	ggc	ttc	cag	gcg	gag	2354	
Leu	Phe	Asn	Leu	Leu	Val	Ala	Ile	Leu	Val	Glu	Gly	Phe	Gln	Ala	Glu		
			690					695					700				
ggc	gat	gcc	aac	aga	tcc	gac	acg	gac	gag	gac	aag	acg	tcg	gtc	cac	2402	
Gly	Asp	Ala	Asn	Arg	Ser	Asp	Thr	Asp	Glu	Asp	Lys	Thr	Ser	Val	His		
	705						710					715					
ttc	gag	gag	gac	ttc	cac	aag	ctc	aga	gaa	ctc	cag	acc	aca	gag	ctg	2450	
Phe	Glu	Glu	Asp	Phe	His	Lys	Leu	Arg	Glu	Leu	Gln	Thr	Thr	Glu	Leu		
	720					725					730						
aag	atg	tgt	tcc	ctg	gcc	gtg	acc	ccc	aac	ggg	cac	ctg	gag	gga	cga	2498	
Lys	Met	Cys	Ser	Leu	Ala	Val	Thr	Pro	Asn	Gly	His	Leu	Glu	Gly	Arg		
	735				740					745					750		
ggc	agc	ctg	tcc	cct	ccc	ctc	atc	atg	tgc	aca	gct	gcc	acg	ccc	atg	2546	
Gly	Ser	Leu	Ser	Pro	Pro	Leu	Ile	Met	Cys	Thr	Ala	Ala	Thr	Pro	Met		
				755					760					765			
cct	acc	ccc	aag	agc	tca	cca	ttc	ctg	gat	gca	gcc	ccc	agc	ctc	cca	2594	
Pro	Thr	Pro	Lys	Ser	Ser	Pro	Phe	Leu	Asp	Ala	Ala	Pro	Ser	Leu	Pro		
			770					775					780				
gac	tct	cgg	cgt	ggc	agc	agc	agc	tcc	ggg	gac	ccg	cca	ctg	gga	gac	2642	
Asp	Ser	Arg	Arg	Gly	Ser	Ser	Ser	Ser	Gly	Asp	Pro	Pro	Leu	Gly	Asp		
		785					790					795					
cag	aag	cct	ccg	gcc	agc	ctc	cga	agt	tct	ccc	tgt	gcc	ccc	tgg	ggc	2690	
Gln	Lys	Pro	Pro	Ala	Ser	Leu	Arg	Ser	Ser	Pro	Cys	Ala	Pro	Trp	Gly		
	800					805					810						
ccc	agt	ggc	gcc	tgg	agc	agc	cgg	cgc	tcc	agc	tgg	agc	agc	ctg	ggc	2738	
Pro	Ser	Gly	Ala	Trp	Ser	Ser	Arg	Arg	Ser	Ser	Trp	Ser	Ser	Leu	Gly		
	815				820					825				830			
cgt	gcc	ccc	agc	ctc	aag	cgc	cgc	ggc	cag	tgt	ggg	gaa	cgt	gag	tcc	2786	
Arg	Ala	Pro	Ser	Leu	Lys	Arg	Arg	Gly	Gln	Cys	Gly	Glu	Arg	Glu	Ser		
				835					840					845			
ctg	ctg	tct	ggc	gag	ggc	aag	ggc	agc	acc	gac	gac	gaa	gct	gag	gac	2834	
Leu	Leu	Ser	Gly	Glu	Gly	Lys	Gly	Ser	Thr	Asp	Asp	Glu	Ala	Glu	Asp		
			850					855					860				

ggc agg gcc gcg ccc ggg ccc cgt gcc acc cca ctg cgg cgg gcc gag Gly Arg Ala Ala Pro Gly Pro Arg Ala Thr Pro Leu Arg Arg Ala Glu 865 870 875	2882
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tgc cgc gat cgc gac ggg cag gtg gtg gcc ctg ccc agc gac ttc ttc Cys Arg Asp Arg Asp Gly Gln Val Val Ala Leu Pro Ser Asp Phe Phe 895 900 905 910	2978
ctg cgc atc gac agc cac cgt gag gat gca gcc gag ctt gac gac gac Leu Arg Ile Asp Ser His Arg Glu Asp Ala Ala Glu Leu Asp Asp Asp 915 920 925	3026
tgc gag gac agc tgc tgc ctg cgc ctg cat aaa gtg ctg gag ccc tac Ser Glu Asp Ser Cys Cys Leu Arg Leu His Lys Val Leu Glu Pro Tyr 930 935 940	3074
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tcc cca cag aac cgg ttc cgc gtc tcc tgc cag aag gtc atc aca cac Ser Pro Gln Asn Arg Phe Arg Val Ser Cys Gln Lys Val Ile Thr His 960 965 970	3170
aag atg ttt gat cac gtg gtc ctg gtc ttc atc ttc ctg aac tgc gtc Lys Met Phe Asp His Val Val Leu Val Phe Ile Phe Leu Asn Cys Val 975 980 985 990	3218
acc atc gcc ctg gag agg cct gac att gac ccc ggc agc acc gag cgg Thr Ile Ala Leu Glu Arg Pro Asp Ile Asp Pro Gly Ser Thr Glu Arg 995 1000 1005	3266
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gag atg atg gtg aag gtg gtg gcc ctg ggg ctg ctg tcc ggc gag cac Glu Met Met Val Lys Val Val Ala Leu Gly Leu Leu Ser Gly Glu His 1025 1030 1035	3362
gcc tac ctg cag agc agc tgg aac ctg ctg gat ggg ctg ctg gtg ctg Ala Tyr Leu Gln Ser Ser Trp Asn Leu Leu Asp Gly Leu Leu Val Leu 1040 1045 1050	3410
gtg tcc ctg gtg gac att gtc gtg gcc atg gcc tgc gct ggt ggc gcc Val Ser Leu Val Asp Ile Val Val Ala Met Ala Ser Ala Gly Gly Ala 1055 1060 1065 1070	3458
aag atc ctg ggt gtt ctg cgc gtg ctg cgt ctg ctg cgg acc ctg cgg Lys Ile Leu Gly Val Leu Arg Val Leu Arg Leu Leu Arg Thr Leu Arg 1075 1080 1085	3506
cct cta agg gtc atc agc cgg gcc ccg ggc ctg aag ctg gtg gtg gag	3554

Pro	Leu	Arg	Val	Ile	Ser	Arg	Ala	Pro	Gly	Leu	Lys	Leu	Val	Val	Glu		
			1090					1095					1100				
acg	ctg	ata	tcg	tcg	ctc	agg	ccc	att	ggg	aac	atc	gtc	ctc	atc	tgc	3602	
Thr	Leu	Ile	Ser	Ser	Leu	Arg	Pro	Ile	Gly	Asn	Ile	Val	Leu	Ile	Cys		
		1105					1110					1115					
tgc	gcc	ttc	ttc	atc	att	ttt	ggc	atc	ttg	ggt	gtg	cag	ctc	ttc	aaa	3650	
Cys	Ala	Phe	Phe	Ile	Ile	Phe	Gly	Ile	Leu	Gly	Val	Gln	Leu	Phe	Lys		
	1120					1125					1130						
ggg	aag	ttc	tac	tac	tgc	gag	ggc	ccc	gac	acc	agg	aac	atc	tcc	acc	3698	
Gly	Lys	Phe	Tyr	Tyr	Cys	Glu	Gly	Pro	Asp	Thr	Arg	Asn	Ile	Ser	Thr		
	1135				1140				1145					1150			
aag	gca	cag	tgc	cgg	gcc	gcc	cac	tac	cgc	tgg	gtg	cga	cgc	aag	tac	3746	
Lys	Ala	Gln	Cys	Arg	Ala	Ala	His	Tyr	Arg	Trp	Val	Arg	Arg	Lys	Tyr		
			1155					1160						1165			
aac	ttc	gac	aac	ctg	ggc	cag	gcc	ctg	atg	tcg	ctg	ttc	gtg	ctg	tca	3794	
Asn	Phe	Asp	Asn	Leu	Gly	Gln	Ala	Leu	Met	Ser	Leu	Phe	Val	Leu	Ser		
		1170					1175						1180				
tcc	aag	gat	gga	tgg	gtg	aac	atc	atg	tac	gac	ggg	ctg	gat	gcc	gtg	3842	
Ser	Lys	Asp	Gly	Trp	Val	Asn	Ile	Met	Tyr	Asp	Gly	Leu	Asp	Ala	Val		
	1185					1190					1195						
ggt	gtc	gac	cag	cag	cct	gtg	cag	aac	cac	aac	ccc	tgg	atg	ctg	ctg	3890	
Gly	Val	Asp	Gln	Gln	Pro	Val	Gln	Asn	His	Asn	Pro	Trp	Met	Leu	Leu		
	1200				1205						1210						
tac	ttc	atc	tcc	ttc	ctg	ctc	atc	gtc	agc	ttc	ttc	gtg	ctc	aac	atg	3938	
Tyr	Phe	Ile	Ser	Phe	Leu	Leu	Ile	Val	Ser	Phe	Phe	Val	Leu	Asn	Met		
	1215				1220				1225					1230			
ttc	gtg	ggc	gtc	gtg	gtc	gag	aac	ttc	cac	aag	tgc	cgg	cag	cac	cag	3986	
Phe	Val	Gly	Val	Val	Val	Glu	Asn	Phe	His	Lys	Cys	Arg	Gln	His	Gln		
			1235				1240						1245				
gag	gcg	gag	gag	gcg	cgg	cgg	cga	gag	gag	aag	cgg	ctg	cgg	cgc	cta	4034	
Glu	Ala	Glu	Glu	Ala	Arg	Arg	Arg	Glu	Glu	Lys	Arg	Leu	Arg	Arg	Leu		
		1250					1255					1260					
gag	agg	agg	cgc	agg	agc	act	ttc	ccc	agc	cca	gag	gcc	cag	cgc	cgg	4082	
Glu	Arg	Arg	Arg	Arg	Ser	Thr	Phe	Pro	Ser	Pro	Glu	Ala	Gln	Arg	Arg		
	1265					1270					1275						
ccc	tac	tat	gcc	gac	tac	tcg	ccc	acg	cgc	cgc	tcc	att	cac	tcg	ctg	4130	
Pro	Tyr	Tyr	Ala	Asp	Tyr	Ser	Pro	Thr	Arg	Arg		Ile	His	Ser	Leu		
	1280					1285					1290						
tgc	acc	agc	cac	tat	ctc	gac	ctc	ttc	atc	acc	ttc	atc	atc	tgt	gtc	4178	
Cys	Thr	Ser	His	Tyr	Leu	Asp	Leu	Phe	Ile	Thr	Phe	Ile	Ile	Cys	Val		
	1295				1300				1305					1310			
aac	gtc	atc	acc	atg	tcc	atg	gag	cac	tat	aac	caa	ccc	aag	tcg	ctg	4226	
Asn	Val	Ile	Thr	Met	Ser	Met	Glu	His	Tyr	Asn	Gln	Pro	Lys	Ser	Leu		
			1315					1320					1325				

gac gag gcc ctc aag tac tgc aac tac gtc ttc acc atc gtg ttt gtc 4274  
 Asp Glu Ala Leu Lys Tyr Cys Asn Tyr Val Phe Thr Ile Val Phe Val  
 1330 1335 1340

ttc gag gct gca ctg aag ctg gta gca ttt ggg ttc cgt cgg ttc ttc 4322  
 Phe Glu Ala Ala Leu Lys Leu Val Ala Phe Gly Phe Arg Arg Phe Phe  
 1345 1350 1355

aag gac agg tgg aac cag ctg gac ctg gcc atc gtg ctg ctg tca ctc 4370  
 Lys Asp Arg Trp Asn Gln Leu Asp Leu Ala Ile Val Leu Leu Ser Leu  
 1360 1365 1370

atg ggc atc acg ctg gag gag ata gag atg agc gcc gcg ctg ccc atc 4418  
 Met Gly Ile Thr Leu Glu Glu Ile Glu Met Ser Ala Ala Leu Pro Ile  
 1375 1380 1385 1390

aac ccc acc atc atc cgc atc atg cgc gtg ctt cgc att gcc cgt gtg 4466  
 Asn Pro Thr Ile Ile Arg Ile Met Arg Val Leu Arg Ile Ala Arg Val  
 1395 1400 1405

ctg aag ctg ctg aag atg gct acg ggc atg cgc gcc ctg ctg gac act 4514  
 Leu Lys Leu Leu Lys Met Ala Thr Gly Met Arg Ala Leu Leu Asp Thr  
 1410 1415 1420

gtg gtg caa gct ctc ccc cag gtg ggg aac ctg ggc ctt ctt ttc atg 4562  
 Val Val Gln Ala Leu Pro Gln Val Gly Asn Leu Gly Leu Leu Phe Met  
 1425 1430 1435

ctc ctg ttt ttt atc tat gct gcg ctg gga gtg gag ctg ttc ggg agg 4610  
 Leu Leu Phe Phe Ile Tyr Ala Ala Leu Gly Val Glu Leu Phe Gly Arg  
 1440 1445 1450

ctg gag tgc agt gaa gac aac ccc tgc gag ggc ctg agc agg cac gcc 4658  
 Leu Glu Cys Ser Glu Asp Asn Pro Cys Glu Gly Leu Ser Arg His Ala  
 1455 1460 1465 1470

acc ttc agc aac ttc ggc atg gcc ttc ctc acg ctg ttc cgc gtg tcc 4706  
 Thr Phe Ser Asn Phe Gly Met Ala Phe Leu Thr Leu Phe Arg Val Ser  
 1475 1480 1485

acg ggg gac aac tgg aac ggg atc atg aag gac acg ctg cgc gag tgc 4754  
 Thr Gly Asp Asn Trp Asn Gly Ile Met Lys Asp Thr Leu Arg Glu Cys  
 1490 1495 1500

tcc cgt gag gac aag cac tgc ctg agc tac ctg ccg gcc ctg tcg ccc 4802  
 Ser Arg Glu Asp Lys His Cys Leu Ser Tyr Leu Pro Ala Leu Ser Pro  
 1505 1510 1515

gtc tac ttc gtg acc ttc gtg ctg gtg gcc cag ttc gtg ctg gtg aac 4850  
 Val Tyr Phe Val Thr Phe Val Leu Val Ala Gln Phe Val Leu Val Asn  
 1520 1525 1530

gtg gtg gtg gcc gtg ctc atg aag cac ctg gag gag agc aac aag gag 4898  
 Val Val Val Ala Val Leu Met Lys His Leu Glu Glu Ser Asn Lys Glu  
 1535 1540 1545 1550

gca cgg gag gat gcg gag ctg gac gcc gag atc gag ctg gag atg gcg 4946

Ala Arg Glu Asp Ala Glu Leu Asp Ala Glu Ile Glu Leu Glu Met Ala	
1555 1560 1565	
cag ggc ccc ggg agt gca cgc cgg gtg gac gcg gac agg cct ccc ttg	4994
Gln Gly Pro Gly Ser Ala Arg Arg Val Asp Ala Asp Arg Pro Pro Leu	
1570 1575 1580	
ccc cag gag agt ccg ggc gcc agg gat gcc cca aac ctg gtt gca cgc	5042
Pro Gln Glu Ser Pro Gly Ala Arg Asp Ala Pro Asn Leu Val Ala Arg	
1585 1590 1595	
aag gtg tcc gtg tcc agg atg ctc tcg ctg ccc aac gac agc tac atg	5090
Lys Val Ser Val Ser Arg Met Leu Ser Leu Pro Asn Asp Ser Tyr Met	
1600 1605 1610	
ttc agg ccc gtg gtg cct gcc tcg gcg ccc cac ccc cgc ccg ctg cag	5138
Phe Arg Pro Val Val Pro Ala Ser Ala Pro His Pro Arg Pro Leu Gln	
1615 1620 1625 1630	
gag gtg gag atg gag acc tat ggg gcc ggc acc ccc ttg ggc tcc gtt	5186
Glu Val Glu Met Glu Thr Tyr Gly Ala Gly Thr Pro Leu Gly Ser Val	
1635 1640 1645	
gcc tct gtg cac tct ccg ccc gca gag tcc tgt gcc tcc ctc cag atc	5234
Ala Ser Val His Ser Pro Pro Ala Glu Ser Cys Ala Ser Leu Gln Ile	
1650 1655 1660	
cca ctg gct gtg tcg tcc cca gcc agg agc ggc gag ccc ctc cac gcc	5282
Pro Leu Ala Val Ser Ser Pro Ala Arg Ser Gly Glu Pro Leu His Ala	
1665 1670 1675	
ctg tcc cct cgg ggc aca gcc cgc tcc ccc agt ctc agc cgg ctg ctc	5330
Leu Ser Pro Arg Gly Thr Ala Arg Ser Pro Ser Leu Ser Arg Leu Leu	
1680 1685 1690	
tgc aga cag gag gct gtg cac acc gat tcc ttg gaa ggg aag att gac	5378
Cys Arg Gln Glu Ala Val His Thr Asp Ser Leu Glu Gly Lys Ile Asp	
1695 1700 1705 1710	
agc cct agg gac acc ctg gat cct gca gag cct ggt gag aaa acc ccg	5426
Ser Pro Arg Asp Thr Leu Asp Pro Ala Glu Pro Gly Glu Lys Thr Pro	
1715 1720 1725	
gtg agg ccg gtg acc cag ggg ggc tcc ctg cag tcc cca cca cgc tcc	5474
Val Arg Pro Val Thr Gln Gly Gly Ser Leu Gln Ser Pro Pro Arg Ser	
1730 1735 1740	
cca cgg ccc gcc agc gtc cgc act cgt aag cat acc ttc gga cag cac	5522
Pro Arg Pro Ala Ser Val Arg Thr Arg Lys His Thr Phe Gly Gln His	
1745 1750 1755	
tgc gtc tcc agc cgg ccg gcg gcc cca ggc gga gag gag gcc gag gcc	5570
Cys Val Ser Ser Arg Pro Ala Ala Pro Gly Gly Glu Glu Ala Glu Ala	
1760 1765 1770	
tcg gac cca gcc gac gag gag gtc agc cac atc acc agc tcc gcc tgc	5618
Ser Asp Pro Ala Asp Glu Glu Val Ser His Ile Thr Ser Ser Ala Cys	
1775 1780 1785 1790	

ccc tgg cag ccc aca gcc gag ccc cat ggc ccc gaa gcc tct ccg gtg Pro Trp Gln Pro Thr Ala Glu Pro His Gly Pro Glu Ala Ser Pro Val 1795 1800 1805	5666
gcc ggc ggc gag cgg gac ctg cgc agg ctc tac agc gtg gac gct cag Ala Gly Gly Glu Arg Asp Leu Arg Arg Leu Tyr Ser Val Asp Ala Gln 1810 1815 1820	5714
ggc ttc ctg gac aag ccg ggc cgg gca gac gag cag tgg cgg ccc tcg Gly Phe Leu Asp Lys Pro Gly Arg Ala Asp Glu Gln Trp Arg Pro Ser 1825 1830 1835	5762
gcg gag ctg ggc agc ggg gag cct ggg gag gcg aag gcc tgg ggc cct Ala Glu Leu Gly Ser Gly Glu Pro Gly Glu Ala Lys Ala Trp Gly Pro 1840 1845 1850	5810
gag gcc gag ccc gct ctg ggt gcg cgc aga aag aag aag atg agc ccc Glu Ala Glu Pro Ala Leu Gly Ala Arg Arg Lys Lys Lys Met Ser Pro 1855 1860 1865 1870	5858
ccc tgc atc tcg gtg gaa ccc cct gcg gag gac gag ggc tct gcg cgg Pro Cys Ile Ser Val Glu Pro Pro Ala Glu Asp Glu Gly Ser Ala Arg 1875 1880 1885	5906
ccc tcc gcg gca gag ggc ggc agc acc aca ctg agg cgc agg acc ccg Pro Ser Ala Ala Glu Gly Gly Ser Thr Thr Leu Arg Arg Arg Thr Pro 1890 1895 1900	5954
tcc tgt gag gcc acg cct cac agg gac tcc ctg gag ccc aca gag ggc Ser Cys Glu Ala Thr Pro His Arg Asp Ser Leu Glu Pro Thr Glu Gly 1905 1910 1915	6002
tca ggc gcc ggg ggg gac cct gca gcc aag ggg gag cgc tgg ggc cag Ser Gly Ala Gly Gly Asp Pro Ala Ala Lys Gly Glu Arg Trp Gly Gln 1920 1925 1930	6050
gcc tcc tgc cgg gct gag cac ctg acc gtc ccc agc ttt gcc ttt gag Ala Ser Cys Arg Ala Glu His Leu Thr Val Pro Ser Phe Ala Phe Glu 1935 1940 1945 1950	6098
ccg ctg gac ctc ggg gtc ccc agt gga gac cct ttc ttg gac ggt agc Pro Leu Asp Leu Gly Val Pro Ser Gly Asp Pro Phe Leu Asp Gly Ser 1955 1960 1965	6146
cac agt gtg acc cca gaa tcc aga gct tcc tct tca ggg gcc ata gtg His Ser Val Thr Pro Glu Ser Arg Ala Ser Ser Ser Gly Ala Ile Val 1970 1975 1980	6194
ccc ctg gaa ccc cca gaa tca gag cct ccc atg ccc gtc ggt gac ccc Pro Leu Glu Pro Pro Glu Ser Glu Pro Pro Met Pro Val Gly Asp Pro 1985 1990 1995	6242
cca gag aag agg cgg ggg ctg tac ctc aca gtc ccc cag tgt cct ctg Pro Glu Lys Arg Arg Gly Leu Tyr Leu Thr Val Pro Gln Cys Pro Leu 2000 2005 2010	6290
gag aaa cca ggg tcc ccc tca gcc acc cct gcc cca ggg ggt ggt gca	6338

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